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PREFACE

In introducing the third volume of the *Review* to its readers it is only fitting that we should gratefully acknowledge the favorable reception which its predecessors have enjoyed. We feel encouraged to believe that the *Review* is beginning to serve effectively the purposes for which it was initiated.

Nevertheless we are acutely aware of a number of formidable difficulties, not the least of which is to compass adequately within 500 or 600 pages the literature in the expanding field of biochemistry. In some instances the reviewer has found it possible to include within his survey of a subject only a third or a fourth of the papers which might be deemed worthy of mention if the limitations upon length were less rigorous. But even at the expense of omitting reference to many papers, we are disposed to believe that critical surveys of the literature, though less comprehensive, are of more value to users of the *Review* than uncritical compendia.

Adoption of this policy imposes upon the reviewer the very difficult task of selecting the papers from which is to be woven his survey of the subject. Overlooking the obvious fact that many papers are necessarily omitted because of their doubtful quality and others are inadvertently neglected, it is but fair to mention that an even greater number are excluded because they pertain to certain phases of the subject which in the judgment of the reviewer might be treated more satisfactorily in a later volume or by another reviewer.

Indeed, certain of the subjects which by tradition and sustained research constitute substantial chapters in biochemistry are themselves so broad that it is not feasible within a brief review to treat satisfactorily each and every aspect. Some problems of great biochemical interest penetrate far into the sister sciences of physiology, anatomy, and physical and organic chemistry. Since more than one of the subjects embraced by the *Review* enjoy this diversity of interest and approach, the task of the reviewer becomes increasingly difficult. It is hoped that the policy of fluidity in authorship to which we have turned will not only lighten the burden upon each contributor but will permit expression of that diversity of interest and outlook which characterizes investigation in any of the numerous fields of biochemistry.

We have also decided to include from year to year occasional reviews on topics of timely nature in which a lively interest has recently developed and significant advances been made. In consequence the

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present volume includes a review on the biochemistry of malignant tissue and another on biochemical studies in the field of dentistry. So also Volume IV is expected to contain a review on choline and related compounds, and one on growth substances in plants.

It is convenient to distinguish such topics clearly from those others which by universal consent constitute the traditional divisions of the subject. These will continue to receive review at annual or biennial intervals.

To add to the quality and usefulness of the *Review* we feel obliged again to request of its readers advice and critical suggestions on matters of authorship, content, and general policy. We cannot refrain from expressing here our indebtedness to those who have been good enough to write to us on such matters. We would also mention appreciatively the courteous aid extended by many laboratories in supplying the reviewers with reprints of their recent publications.

It is with a deep sense of obligation that we associate ourselves with the many readers of the *Review* who have had occasion to feel especially grateful to the reviewers for their labors. Whatever merit the *Review* possesses is due entirely to their generous co-operation.

To the Stanford University Press we continue to be indebted for its patience and care in composition, for the excellence of its workmanship, and for its cordial co-operation throughout.

J. M. L.
C. L. A.
D. R. H.
C. L. A. S.

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WATER IN ITS BIOCHEMICAL RELATIONSHIPS*

By Ross Aiken Gortner

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From the number of titles, dealing more or less directly with the rôle of water in living processes, which have appeared in the literature during the past two years, it is apparent that biochemists and physiologists are devoting an increasing amount of attention to this important subject.

It will be impossible, within the space allotted to this review, to abstract adequately all of the literature which has appeared. The most which can be done will be to present what it is hoped will be a fairly complete bibliography of those papers dealing primarily with water relationships and to review briefly those which appear either to contribute new viewpoints or technics or to extend our knowledge in some important aspect of the field.

General Reviews

It is perhaps fortunate that several other general reviews covering certain subdivisions of the field of the present paper have appeared in the recent literature, for these can be consulted as supplementary to the subject. Thus Adolph (1) has discussed "The Metabolism and Distribution of Water in Body and Tissues" (340 references); McQuarrie (2), "Significance of the Water Metabolism in Health and Disease" (122 references); Wigglesworth (3), "The Rôle of Water in the Physiology of Excretion in Insects"; Peters & Lavietes (4), "The Nature of Preformed Water"; Meyer (5), "The Colloidal Osmotic Pressure of Biological Fluids"; and Stiles (6), "Recent Advances in Science: Plant Physiology," in which the water relationships of the plant are reviewed.

Physiological Effects Due to the Different Molecular Forms of Water

The physical properties of liquid water are not consistent with the simple "mono-hydrol" (H_2O) formula, and it has been generally

* Received January 12, 1934.

recognized that polymers of water molecules must be present. Di-hydrol, $(H_2O)_2$, and tri-hydrol, $(H_2O)_3$, groupings have been usually regarded as the predominant molecular forms in water at room temperature, with the ice form being largely, if not exclusively, tri-hydrol [Barnes (7)]. Rao (8), from a study of the Raman spectrum, gives the following distribution of H_2O, $(H_2O)_2$, and $(H_2O)_3$ molecules for water at different temperatures: ice, 0, 41, and 59 per cent, respectively; water at 0°, 19, 58, and 23 per cent; water at 38°, 29, 50, and 21 per cent; water at 98°, 36, 51, and 13 per cent. Barnes (9) has studied the growth of *Spirogyra* in water derived from melting ice, in which he states the prevailing polymer is tri-hydrol, in water from condensed steam (predominantly mono-hydrol), and in water which has stood for some time at room temperature (predominantly di-hydrol). His findings are shown in Table I.

TABLE I

THE EFFECT OF WATER POLYMERS ON *Spirogyra**

Temperature ° C.	Light Intensity Ft.-Candles	Predominant Polymer	Dimensions of Colony at Start of Experiment cm.	Dimensions of Colony Four Days Later cm.
10	30	Mono-hydrol	$4 \times 3 \times 0.5$	$5 \times 4 \times 0.75$
10	30	Di-hydrol	$4 \times 3 \times 0.5$	$6 \times 4 \times 1.75$
10	30	Tri-hydrol	$4 \times 3 \times 0.5$	$7 \times 7 \times 2.75$

* Data of Barnes.

Barnes suggests that the polymerized molecules are of primary importance in physiological processes, and may account for the unexpected richness of the microflora of the arctic oceans. Barnes supports the foregoing findings in three later papers (10, 11, 12), the last one dealing with the effects of the water polymers on *Euglena gracilis*, where "mono-hydrol" increased the cell count 32 per cent, whereas "tri-hydrol" increased it 105 per cent in the same time interval. In this same paper Barnes replies to the criticisms of Menzies (13) regarding the polymeric forms of water.

The discovery of the hydrogen isotope (Deuterium) of mass 2 and the isolation of quantities of "heavy water," (H^2H^2O), containing this isotope have opened a new field in the physiology of water. Lewis & Macdonald (14, 15) have shown that heavy water has a

higher viscosity, a higher melting-point (+3.8°), a higher boiling-point (101.42°), a greater density (1.1056 at 25°), and a greater heat of vaporization than ordinary water; Lewis, Olson & Maroney (16) have shown that it has a lower dielectric constant; and Lewis & Doody (17) report that the mobilities of ions in heavy water and the mobility of the $(H^2)^+$ ion are different from the generally accepted constants. Lewis (18) finds that tobacco seeds do not germinate in heavy water and that germination is greatly retarded in water containing 50 per cent of deuterium oxide. Taylor, Swingle, Eyring & Frost (19), using water containing 92 per cent of deuterium oxide, find that tadpoles of *Rana clamitans* died within an hour, the fish *Lebistes reticulatus* was killed in two hours, the flatworm *Planaria maculata* survived only three hours, and the protozoan *Paramoecium caudatum* was killed within forty-eight hours. Water containing 30 per cent of deuterium oxide appeared to be relatively without effect on the organisms mentioned above. Barnes (20) reports differential growth of *Spirogyra* in diluted heavy water. Balinkin (21) suggests that the toxic effects may be due to a selective permeability of the plasma membrane which screens out $H_2{}^2O$ molecules, admitting only the proterium oxide, $(H_2{}^1O)$.

The effect of the substitution of the deuterium form of hydrogen into organic molecules in place of ordinary hydrogen should open a new and important field in organic chemistry. While no published results are reported in this field, apparently several laboratories have such projects under way.

"Bound" versus "Free" Water

The concepts involved in the "bound" water hypothesis were covered in the preceding review (22) and accordingly will not be presented again. However, several papers have appeared in the interval which contribute to this subject. Perhaps the most important of these is the study by Sayre (23) in which a comparison is made of the cryoscopic, calorimetric, and dilatometric methods for measuring "bound" water. Sayre discusses each technic in detail and presents explicit directions for carrying out the laboratory procedures. Using an 18.6 per cent gum acacia sol, he finds that all three methods yield very similar results. His comparative data on a series of replicate determinations by each method are shown in Table II. Sayre finds

that the calorimetric method is easy, accurate, and reliable, and recommends its use for practically all materials. Sayre determined the bound water in a series of biological materials and reports the following percentages of the total water as existing in the "bound" condition: February 28, buckeye twigs 52.2 per cent, buckeye buds 54.6 per cent; March 4, maple twigs 53.6 per cent; March 6, pine needles 32.6, 33.7, and 34.8 per cent; April 1, pine needles 35.0 and 28.2 per cent; August 12, corn blade 21.6 and 21.3 per cent, corn sheath 13.3 per cent, corn stem (upper) 11.2 per cent, (middle) 7.8 per cent, and (lower) 7.9 per cent.

TABLE II

COMPARISON OF THE THREE METHODS OF MEASURING BOUND WATER IN AN 18.6 PER CENT GUM ARABIC SOLUTION*

Cryoscopic Method Per Cent	Calorimetric Method Per Cent	Dilatometric Method Per Cent
10.84	10.59	13.11
15.59	11.97	9.17
12.23	14.12	11.80
13.00	11.07	11.32
11.10	13.87	13.68
10.92	15.80	13.59
Mean = 12.28	Mean = 12.90	Mean = 12.11
σ = 1.83	σ = 2.02	σ = 1.74
$PE_m = \pm 0.50$	$PE_m = \pm 0.56$	$PE_m = \pm 0.48$

* Data of Sayre (23).

St. John (24), in a study somewhat similar to that of Jones & Gortner (25) noted in the last review, finds that the free water of a hydrophilic biocolloid is all frozen at —12.5° and that bound water is not frozen at —35°. He reports approximately 26 per cent bound water in the thick portion of egg white.

Bodenheimer & Schmidt (26), as well as Salt & Hinman (27) report on the use of the calorimetric method of the measurement of bound water as related to entomological problems. Stark (28) finds that in plant tissues the specific heat of the material is almost perfectly correlated with the water content so that the necessity of determining the specific heat of the material for use in the calorimetric

formula is eliminated. Nepenin & Khviyuzov (29) find that approximately 30 per cent of the moisture in wood chips is not frozen at —12°.

Gortner & Gortner (30) have replied to the criticisms of Grollman (31) in regard to the determinations of bound water by the cryoscopic method and have made a redetermination of the bound water in 3 per cent and 5 per cent gum-acacia sols. They report that 0.6–0.7 gram bound water is associated with each gram of the gum acacia. It is of interest to note that this value agrees almost exactly with the average bound water (0.66 gram of bound water per gram of gum) found by Sayre (cf. Table II) on an 18.6 per cent gum-acacia sol.

Greenberg & Greenberg (32) studied the concentration of the solute in ultrafiltrates from sols of gelatin, casein, starch, glycogen, and blood serum. As added solutes they used urea, glucose, KCl, NaCl, and Na_2SO_4. They report little or no increase in concentration of the solute in the ultrafiltrate; in fact in a number of instances they report a diminution in concentration of the solute and accordingly negative bound water values. They accordingly conclude that bound water is non-existent. Bull (33), however, points out that the bound water concept has a substantial theoretical basis and that probably the negative findings of Greenberg & Greenberg were due to a relatively slight selective adsorption of the solute. Gortner & Gortner (30) also report selective adsorption of KCl and KBr by gum acacia, this adsorption accounting for negative values of bound water when these salts are present.

Sunderman (34, 35), using cryoscopic technic, finds that practically all of the water in blood serum is "free," and Hetherington (36) concludes that this is true for the total water of the body, whereas Jochims (37) finds a very considerable fraction of the water in blood plasma to be associated with the plasma colloids. Similarly Ernst & Czimber (38), Hurthle (39), and Mennie (40) report appreciable amounts of bound water in muscle tissue.

Huttig (41) has made a study of the dehydration of gels, following the dehydration curves with simultaneous X-ray diffraction studies. He classifies the systems into (*a*) crystalline hydrates with water in stoichiometrical relationships, (*b*) mixed hydrated crystals, (*c*) amorphous hydrates with water in stoichiometrical relations, (*d*) hydrogels with capillary water, (*e*) osmotic water as in zeolites, and (*f*) adsorbed water. A similar study should be made of bio-

colloid gels. Perhaps such a study would assist in reconciling divergent views on the bound ⇆ free water hypothesis.

Water and Winter Hardiness

Maney (42) reports but little correlation between bound water, as determined by the calorimetric method, and hardiness in apple seedlings. However, he does state that hardy varieties in general were characterized by somewhat larger percentages of unfrozen water.

Mudra (43), studying factors involved in the cold-resistance of winter wheat, finds a decreased water content, an increased osmotic pressure, an increased buffering power, and a greatly increased stability of the plasma colloids.

Meyer (44) studied the bound water relationships in the leaves of the pitch pine (*Pinus rigida* Mill.). He finds that evergreen leaves in general are not cold-resistant during the summer months and that hardening begins at about $+6°$ and below this temperature hardening proceeds rapidly. The summer leaves contained about 5 per cent more water than the winter leaves. Larger amounts of sap can be expressed from summer leaves, but the difference is due almost entirely to the higher water content. Using the calorimetric method Meyer found that the ratio of bound to free water apparently did not change significantly from the summer leaves to the winter leaves, and he concludes that in this species cold-resistance is not dependent upon an increase in bound water, suggesting that the basis for cold-resistance lies in some not yet understood physico-chemical property of the protoplasm.

Greathouse (45) studied the effect of various physical environments on the physico-chemical properties of plant saps. Plants which had been subjected to periodic wiltings and then brought back to optimum moisture conditions yielded saps which were more concentrated than check plants. The addition of salt (NaCl) to the soil gave similar effects to periodic wiltings. Cabbage plants were subjected to lowered temperature to initiate the "hardening off" process, and bound water was studied on saps expressed from such plants. A correlation of $r = 0.94 \pm 0.022$ was found between the concentration and osmotic pressure of these saps, a correlation of $r = 0.66 \pm 0.109$ between concentration and bound water, and a

correlation of $r = 0.54 \pm 0.013$ between osmotic pressure and bound water. Greathouse notes that osmotic pressure frequently afforded no indication of the ability of a plant to withstand low temperature, whereas percentage of bound water did give a good index of both drought-resistance and cold-hardiness.

Desiccation and Water Relationships

Wiley & Wiley (46) find that dehydration of man to a loss of 5 per cent of the body weight causes a marked loss of both KCl and NaCl. They conclude that water is first lost from the serum and interstitial fluids and then, if the dehydrated condition is maintained, water is withdrawn from the body cells. On rehydration water is first stored in the cells.

Takai (47) studied the effect of the withdrawal of water on the growth of young rats. The greatest loss in weight occurred when the animals had received a diet containing excessive amounts of protein or which was deficient in salt. The survival was the greatest in those animals fed a high fat diet and least on a high protein or deficient salt diet. Water was lost from the tissues faster in rats fed a diet deficient in salts. Acidosis developed during dehydration, and this was probably caused by the marked decrease which was observed in oxidative activity of the tissues. Takai suggests that the effect of the high protein diet is probably due to the small amount of metabolic water formed by the oxidation of protein, one gram of protein yielding only 0.413 gram metabolic water, whereas one gram of fat yields 1.071 grams.

Carmichael & Rivers (48) studied the effect of dehydration upon the hatchability of eggs of *Limax flavus* Linn. Eggs containing very young embryos were dried until they had lost 80 to 85 per cent of their original weight. These eggs still hatched and the young lived. The same was true of eggs containing full-term embryos which had been desiccated so as to lose 70 to 75 per cent of the original weight. Fully developed embryos could be desiccated to a loss of 30 to 40 per cent of their weight and still survive.

Carmichael (49) finds that guinea pigs from which water had been withheld for 48 hours previous to the injection were much more susceptible to the action of a subcutaneous injection of tetanus toxin than were normal animals.

Herrick (50) studied the variation in osmotic concentration of the cell sap of the aërial portions of *Ambrosia trifida* L. and reports an increasing gradient of osmotic concentration from the hypocotyl to the immature internodes near the apex of the stem. Increasing drought conditions resulted in increasing osmotic values throughout the entire aërial portion of the plant.

Dastur & Desai (51) find that the rate of photosynthesis in leaves is more closely related to the water content than it is to the content of chlorophyll.

Sanna (52) reports an interesting reaction of *Polygonum amphibium* to water. This plant grows either in water or on land. Growing in water it develops smooth leaves containing a maximum of 0.017 per cent alkaloids. Growing on land it develops hairy leaves containing up to 0.290 per cent alkaloids. If plants on land are heavily watered, the leaves become smooth and the alkaloid content almost disappears.

Water and Growth

Orru (53) has studied the changes in the water content of the albumin and yolk of hens' eggs during incubation. He finds that the water content of the albumin falls until the twelfth to the thirteenth day, then increases again to almost its initial value. The water content of the yolk rises until the twelfth day, then falls again but never reaches the low level of the original yolk.

Wilkerson & Gortner (54) made a study of chemical changes occurring during growth of pig embryos. The total water content decreases rapidly from the 4 mm. to the 15 mm. stage, after which a remarkable constancy is maintained until the 160 mm. stage, and then follows a gradual decline in water content until birth, the final water equilibrium being reached only in postnatal life. They suggest that the equilibrium preceding the 160 mm. stage is maintained by the mesonephros, the later decline occurring when the metanephros assumes an excretory function.

Jackson & Smith (55) studied the effect of deficient water intake over a period of several months on the growth of the rat. Food consumption roughly paralleled water intake. There was a differential distribution of water among the various organs of the body. The percentage differences from the normal were approximately as

follows: skin −9.5, spleen −2.8, muscles +0.1, eye balls −2.6, lungs −3.3, heart −0.3, liver +0.2, kidneys −2.4, testes −0.7, brain −0.2.

Paffrath & Massart (56) in a study of water and mineral metabolism in premature infants find that certain minerals (e.g., Ca) are retained without a corresponding retention of water but that this is never true of the alkali bases. Premature infants react like edematous adults.

Cahane (57) finds that the water content of the crystalline lens of cattle decreases with age, whereas the cholesterol content increases.

Meyer (58) studied transpiration in young tulip poplars (*Liriodendron tulipifera* L.). He reports a peak of transpiration occurring a little before midday with very low transpiration during hours of darkness. During a twenty-four-hour period an average of 10 grams of water was transpired per square decimeter of leaf surface, 7.96 grams of water per gram of fresh weight of the plant, and 37.36 grams of water per gram of dry weight.

Water Absorption and Water Transport

Duryee (59) has studied the metabolism of *Planaria dorotocephala* and early embryos of *Amblystoma punctatum* as measured by oxygen consumption of organisms dehydrated by immersion in Ringer's solution or hydrated in distilled water, against controls in tap water. The *Planaria* showed a decreased oxygen consumption of 54 per cent in distilled water and an increase of 43 per cent in Ringer's solution. The *Amblystoma* showed corresponding values of −21 per cent and +62 per cent. The author points out that in studying metabolic rates the state of hydration of the tissues must be taken into consideration.

Wells (60) studied the rate of absorption of water from a dog's intestine and concludes that absorption is due to the colloid osmotic pressure of the tissue fluids of the villi. Depending upon the animal, absorption is abolished at a negative pressure of 8–26 cm. of solution. Smirk (61) found that in man one liter of warm water is completely absorbed in 22–55 minutes after ingestion and that absorption of water from the intestine is much more rapid than is the excretion of water by the kidneys, the maximum rate of urine secretion being attained 20–30 minutes after the maximum degree of blood dilution

has been reached [Smirk (62)]. Heller & Smirk (63) report similar results for the guinea pig, rabbit, and rat, and conclude that the rate of urine formation is not proportional to the co-existing degree of hydration of the muscle, liver, and blood. Koropov (64) likewise presents data to show that water is very rapidly absorbed from the alimentary tract, the absorption being largely through the portal vein into the circulation. The lymphatic system does not participate in the process.

Auguste (65) reports that the ingestion of 500 cc. of distilled water markedly changed the ratio of albumin to globulin in the blood serum, in general the increase in the ratio paralleling that of the hydremia.

Bennhold (66) has presented a general discussion of transport problems in the animal body, noting that the mechanisms affecting the circulation of the blood are only one aspect of the problem. He emphasizes that in addition to these one must consider water transport by the hydrated protein molecules, the transport of cholesterol and other lipids by globulins, of bile pigments by proteins, of oxygen by hemoglobin, etc.

In the field of plant physiology Duncan & Cooke (67) find that the absorption of water by the roots of sugar cane is greatly affected by variations in the intensity of the sunlight, maximum absorption occurring in full sunlight. Absorption at night, even under artificial light, amounted to only about 20 per cent of the minimum absorption during daylight. A lowering of the temperature of the water surrounding the roots from 28° to 10° caused a great decrease in rate of water absorption, and when the temperature was later raised to the original 28° there was a lag of approximately two hours before the absorption rate became characteristic of the higher temperature.

Water Intoxication and Diuresis

Gömöri & Molnár (68) find that the minimum osmotic concentration in the brain, liver, and muscle of rabbits which is compatible with life corresponds to a freezing-point lowering of not less than 0.6°, and they express the belief that water intoxication is not due to increased intracranial pressure but to an excessive lowering of the tissue osmotic pressure. They find that muscle binds most of the

water, the liver binds moderate quantities, the nervous system only small amounts. Smyth, Deamer & Phatak (69) ascribe the convulsions of water intoxication not to the water *per se* but to a loss of chlorine by way of the gastric secretion and a resulting alkalosis. Sacerdoti (70) reports the M.L.D. of distilled water given intravenously to rabbits as 220 cc. per kg. Only above 120 cc. do symptoms appear which indicate an altered renal function. He states that the death which quickly ensues at 220 cc. and above is due not merely to mechanical factors but to a profound physico-chemical alteration of the entire organism.

Osterhout & Hill (71) in working with cells of *Nitella flexilis* find that after these have been two or three days in distilled water they no longer respond to electric stimulation and appear to be anesthetized. They recover when placed in pond water, nutrient solution, or dilute solutions of $CaCl_2$. They suggest that the anesthesia appears to be produced by the removal of something from the cell and that the antagonistic action of $CaCl_2$ appears to prevent this removal. They raise the question as to whether or not other kinds of anesthesia can be explained on this basis. The K:Na antagonism disappears in these anesthetized cells but returns when the cell has been replaced in pond water or nutrient solution. From this observation they suggest that the change in cell protoplasm is probably largely a surface effect.

Klisiecki *et al.* (72) followed the course of water absorption and urine secretion in the dog and find that the peak water load in the tissues precedes by about fifteen minutes the maximum rate of urine secretion. The response of the two kidneys may be somewhat dissimilar, but the response is not influenced by the nerve supply of the kidney. The inhibition of water diuresis by either exercise or posterior pituitary extract is independent of the kidney nerve supply. Thyroxin administered subcutaneously increases the intensity and decreases the duration of the response of the kidney to ingested water.

Molitor & Pick (73) find that diuresis produced by drugs is different from diuresis produced by excessive water ingestion. Drugs (caffeine, theophylline, salyrgan, and novurit) produce diuresis in dogs and rabbits which have not been recently given water, and the drug diuresis is not materially increased if large amounts of water are given *per os* simultaneously.

Moraczewski and co-workers (74), reporting on the Hofmeister

series and diuresis, find that NaCNS causes decreased urine excretion and a marked retention of water in the body with, however, an increased excretion of chlorine. NaI likewise inhibits diuresis. Na_2SO_4, on the opposite end of the lyotropic series, causes increased urinary secretion. Murray (75) reports that the diuretic action of ethyl alcohol is not due to a specific effect on the kidney and that it antagonizes the antidiuretic action of pituitrin. Starkenstein (76) finds that the diuretic action of sucrose is exhibited only by dilute solutions, the dilute solutions of sucrose behaving like hypotonic salt solutions, whereas more concentrated solutions behave more like isotonic salt solutions. The rate with which the sugar is metabolized will greatly influence its diuretic behavior.

Page (77) has studied the action of certain diuretics on kidney function as measured by the urea clearance test and reports that caffeine, salyrgan, and diuretin do not alter the ability of the kidney to secrete urea in hypertensive and nephritic patients.

Water and Mineral Balances in Epilepsy

McQuarrie and co-workers (78, 79, 80, 81, 82, 83, 84, 85) have continued studies on the biochemistry of epilepsy. Three of these papers (83, 84, 85) are more or less of the nature of general reviews. Space perhaps can be conserved by summarizing not only these papers but other contributions in this field by a quotation from one of these reviews (85).

> The fact, that a large percentage of epileptic patients do not show structural changes in the central nervous system sufficient to account for their bizarre symptoms, has led investigators to seek for the cause of the disease in disturbances of brain-cell physiology. That some abnormality in the metabolism of the brain actually occurs is suggested by the fact that convulsions usually cease during a prolonged period of fasting or when the victim of the disease is given a ketogenic diet or is placed on any regimen which produces a deficit in the body water. As a general rule, the factors which tend to increase cell membrane permeability (alkalosis, superhydration, anoxia, excitation, hyperlecithinemia) are those recognized as favoring the occurrence of epileptic seizures; whereas those which are thought to decrease permeability (narcosis, acidosis, dehydration, ketosis, hypercholesterinemia) favor their cessation. Recent biochemical studies on the mineral and water balances in relation to the occurrence of epileptic convulsions are tentatively interpreted as indicating the existence of an inherent defect in the physiological mechanism for regulating the semi-permeability of the brain-cell membranes.

Lyon & Dunlop (86) have produced major seizures by forced intake of fluid or inhibition of diuresis by pituitrin. The administration of alkalies caused an increase in the frequency of seizures coincident with a rise in the alkali reserve of the blood. Periods of dehydration produced beneficial effects in a great majority of cases. Dehydration or a ketogenic diet produced approximately equally favorable results but were not found to be as efficient as administration of luminal. The effects of luminal, however, could be enhanced by maintaining a state of dehydration. Byrom (87) and Proescher & Thomas (88) also have studied water and mineral balances in epileptic patients, and their reports indicate that abnormal mineral balances may be of more importance than the water relationships.

Water Relationships in Physiological Processes

Water exchange.—Adolph (89) has published a general review of "Exchanges of Water in the Frog" in which 73 references are cited. He points out that the frog's high rate of water absorption is only a fraction of the rate which would characterize a similar sac of isotonic salt solution and that the frog's resistance to osmosis is localized in the nervous system, since the resistance to the free entrance of water is abolished either by removal of the skin or by pithing the brain. Studies on the isolated skin fail to account for the different rates of water absorption which the intact frog exhibits under diverse conditions. Adolph (90) notes that urine secretion by the frog's kidney ceases in the absence of oxygen and that this failure of secretion is due to a cessation of blood flow through the glomeruli, other portions of the kidney continuing to receive a copious supply of blood.

Whitehouse, Hancock & Haldane (91) have studied moisture lost through the human skin. Osmosis and diffusion account for the water transfer. The transfer of gases (CO_2 and O_2) roughly parallels the movement of water.

Water and mineral exchange have been studied by Manchester (92) on two slightly epileptic boys. Peters, Kydd & Lavietes (93) and Newburgh, Wiley & Johnston (94) have contributed to the problem of the method of calculation of the water exchange.

Salivary flow.—Gregersen & Bullock (95) studied salivary flow and blood volume in man following two days of water deprivation. Salivary flow fell from a normal of 5 cc. in 5 minutes to 0.7 cc.;

plasma volume fell from 3000 cc. to 2240 cc. There was an increase in erythrocytes and a concentration of the plasma proteins with urine excretion falling to less than 12 cc. per hour.

Adolph, Gerbasi & Lepore (96) report that hemorrhage is immediately followed by a great dilution of the blood plasma and, by clamping off the aorta and vena cava above the diaphragm, showed that most of the diluting fluid came from the viscera. They ascribe the flow of water from the viscera into the plasma to a lowered capillary pressure.

Colloid osmotic pressure.—The rôle of the plasma colloid osmotic pressure in maintaining the water balance between the blood and body tissues has been investigated by Del Baere (97), and Wells, Youmans & Miller (98). The latter authors present a nomogram to facilitate the calculation of the colloid osmotic pressure from the albumin and protein content of the sera.

Water and Hormone Relationships

The pituitary.—Heller & Smirk (99), Smirk (100), Gaebler (101), Goldzieher & Kaldor (102), Steggerda & Freedman (103), Robert (104), and Grassheim (105) have all contributed to the problem of the water relations of the body as affected by extracts of the pituitary. They all agree in finding an increased water retention by the body tissues and a lowered urine secretion.

The adrenals.—Swingle *et al.* (106), Silvette (107), and Winter & Hartman (108) all report studies dealing with the effect of the adrenal cortical hormone on the water relations within the body. In the absence of this hormone fluid is lost from the circulation, and the skin, liver, and muscle tissues become more highly hydrated than normal. Adrenalectomized animals die from circulatory collapse due to an insufficiency of circulatory fluid, for in the absence of the cortical hormone they are unable to draw fluid back into the blood stream through the capillary walls. In the absence of the cortical hormone, urine excretion falls markedly, and the rate of urea elimination is greatly decreased.

Glycogen and Water Storage in the Liver

Puckett & Wiley (109) and MacKay & Bergman (110) present evidence that from 2.4 to 3.0 grams of water are stored in the

liver along with each gram of glycogen. These findings are opposed by Holmquist (111) and Bridge & Bridges (112) who argue that there is no relationship between the water content and the glycogen content of the liver.

The Action of Chemicals and Drugs on Water Relationships

Lecithin and cholesterol.—Dahmlos (113) finds that lecithin and cholesterol are antagonistic in regard to their behavior toward the distribution of water and inorganic ions between the blood and tissues and that the behavior depends upon whether they are injected into the portal or into the jugular vein. Injected into the portal vein they are largely retained in the liver, whereas if they are injected into the jugular vein, they remain to a great extent in the general circulation. Dahmlos injected these lipids under a variety of conditions and found that the mode of injection had a great influence upon the subsequent water and chloride distribution between blood and tissues.

Sodium chloride.—Collins (114) studied the effect of intravenously injecting Ringer-Locke's solution in amounts up to 25 per cent of the animal's weight on the production of fluid in serous cavities. The dogs were fasted for two days previous to the experiment and were given no water for twelve hours preceding the experiment. In the absence of kidney secretion considerable ascitic fluid was produced but only slight amounts of fluid in the pleural or pericardial cavities. Even with large saline injections the greater part of the fluid did not remain long in the blood stream.

Calvin, Smith & Mendel (115) injected a volume of isotonic salt solution equivalent to the calculated blood volume into the femoral vein in less than five minutes and studied the composition of the blood samples subsequently withdrawn. The injection did not result in a simple dilution of the blood. The injected chloride was removed from the blood stream at a faster rate than water. Later the rates were reversed. The urine excretion at first contained relatively more water than chlorides, later relatively more chlorides than water. The authors note that the plasma may act as a water depot for as long as four hours following injection under their conditions.

Muller (116) notes that patients suffering from pulmonary tu-

berculosis on a salt-free diet retain water in their tissues, but when sodium chloride is added to the diet the retained water is eliminated. Serum proteins in cirrhotic tuberculosis decrease following the ingestion of sodium chloride, indicating a dilution of the blood, whereas in exudative tuberculosis serum proteins increase with ingestion of sodium chloride.

Fever-producing drugs.—Marshall & Barbour (117) find that the fever induced by β-tetrahydronaphthylamine, or anaphylactic fever, or fever induced by the administration of Shiga vaccine [Horwitt, Sherman & Barbour (118)] are all accompanied by liver hydration, as was the case in cocaine fever previously reported. Amidopyrine in large doses acting as an antipyretic did not alter the water content of the liver.

Pyramidone.—Scherf (119) finds that patients given two grams of pyramidone daily develop salt and water retention which may amount to as much as 0.5 kg. and which is not compensated for by the circulation. Pyramidone inhibits diuresis caused by digitalis and purines and usually inhibits that caused by salyrgan. Withdrawal of the drug is followed by the excretion of the retained salt and water.

Anesthetics and hypnotics.—Heller & Smirk (120) studied the effect of anesthetics and hypnotics on the absorption and excretion of water in the rat. Absorption of water from the intestine was delayed by ether and chloroform, to a lesser extent by luminal, urethan, and paraldehyde. It was not affected by chloralose. Diuresis was inhibited by ether and chloroform. Barron (121) finds that there is a marked sex difference in the reaction of albino rats to sodium amytal, the male rats being much less sensitive than the female rats. He finds that the resistance to sodium amytal is in some way related to the water metabolism of the animal. Animals which had received a lethal dose of sodium amytal could be revived by injecting Ringer's solution into the blood stream.

The effects of chlorides, bromides, and fluorides on the water relationships of the plant.—Wilson (122) has grown tobacco plants under glass in the presence of the sodium salts of these halogen acids. All of these salts increased the osmotic concentration of the plant sap, and the increased osmotic concentration was correlated with an increase in the size of the leaf cells. The leaves of the tobacco plants to which excessive amounts of chlorides or bromides had been added contained less bound water than leaves of other plants grown in a medium deficient in mineral nutrients. Tobacco plants grown in the

presence of sodium fluoride transformed free water into bound water in such quantities as to indicate that this compound induces a state of physiological drought.

Water Relationships in Pathological Conditions

Nephritis and nephrosis.—Lashmet & Newburgh (123) find that in individuals with renal damage there are two types of response to water restriction. With nephritis but without nephrosis the urine volume is still large at the expense of body water; with nephrosis urine is small because of the affinity of the body tissues for water.

Hatafuku (124) finds that even in the absence of edema large amounts of fluid may be retained in the tissues of nephrotic patients, and that the tissues are able to concentrate a protein-free edema fluid to a fluid containing even more protein than blood serum.

Edema.—Muntwyler *et al.* (125) studied the plasma-colloid osmotic pressure on 10 normal and 42 hospital patients. They find that edema is generally absent when the plasma-colloid osmotic pressure exceeds 20 cm. of plasma. In normal subjects the pressure lies between 29 and 36 cm. of plasma.

Cancer.—Schlottmann & Rubenow (126) find an increase of +2.1 per cent in the water content of the blood of rats with carcinoma and +3.0 per cent in the blood of those with sarcomas. The water content of the muscles, kidneys, and brain was below normal, that of the spleen was unchanged, while that of the liver increased as in the blood. Tumor tissue resembles embryonic tissue in its high water content.

Protoplasm

Huber (127) finds that plasmolysis is 30–40 times slower in mesophyll cells of *Vallisneria spiralis* than in epidermal cells and ascribes this difference to a difference in the permeability to water of the protoplasm in these cells. De Haan (128) has presented an extensive study on "The Swelling of Protoplasm and Water Permeability" (114 references). The length of the paper and the variety of topics discussed are such that a brief review is impracticable, so that the original must be consulted for details.

The Hydration and Dehydration of Gels

The following papers, while not dealing directly with the living organism, nevertheless contain either technics or information which may assist the biochemist and physiologist in the solution of problems involving water relationships: Sigon (129), "The State of Hydration of the Serum Proteins"; du Noüy (130, 131), "The Blood Serum," and "The Electrical Conductivity of Serum as a Function of Temperature"; Barker (132), "The Effect of Water Content upon the Rate of Heat Denaturation of Crystallizable Egg Albumin"; Moran (133), "Hydration or Combined Water of Gelatin"; Jordan-Lloyd (134), "Absorption of Water by Gelatin VI"; Champetier (135), "Fixation of Water by Cellulose"; Gortner (136), "The Hydration Capacity of Starch"; Katz & Derksen (137), "The Change in X-ray Spectrum of Inulin on Swelling in Water"; Prakash (138), "Surface-Combined and Structurally Combined Water in Inorganic Gels"; and Rossi & Marescotti (139), "Syneresis."

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BIOLOGICAL OXIDATIONS AND REDUCTIONS*

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Oxidation-Reduction Potentials

Within recent years the potentials of a large number of dyestuffs have been measured, especially by Clark and Conant and their schools. The latest publications deal, among other things, with the extremely positive indophenol systems (1) and oxazines (2).

Michaelis (3) brought forward the first example of a reversible two-stage oxidation-reduction system. This was followed by the discovery in nature of such a system, when the potential of chlororaphin, the green colouring matter from *Bacillus chloraphis,* was measured (4). In order to explain the results obtained in the titration of reduced chlororaphin with oxidising agents, a two-stage oxidation must be assumed; green chlororaphin, the half-reduced intermediate product, must therefore be formulated as a semiquinone.

Use can also be made of the microinjection of oxidation-reduction indicators to measure the potentials existing in living cells, as well as the potentiometric method. Needham & Needham, Wurmser, and others have carried out such investigations. A value of 200 mv., corresponding to a rH value of 20, has been obtained for aërated cells. Among other things, it is important to decide if a genuine oxidation-reduction equilibrium does really exist in the living cell. The presence of oxygen complicates matters, as it can interfere with the oxidation-reduction system of the cell. From their study of the oxidation-reduction systems connected with glucose, Wurmser & Geloso (5) have come to the conclusion that such reversible systems do occur in the cell, that they are interdependent, and that they act as suppliers of hydrogen for the syntheses in the living cell which are, in the main, reduction processes. This may hold good from time to time. If the oxidation-reduction potential remains constant, then clearly only one substance can be used, which does not give rise to a thermodynamically reversible system, for, were it otherwise, the oxidation-reduction potential would alter considerably as soon as a small portion was transformed into the oxidised form. On the other hand, however,

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were an irreversible system present, even a considerable oxidation would have relatively little influence on the potential. Ascorbic acid (vitamin C) (6) is, after glutathione, the best-known constituent of cell-juice whose oxidation-reduction potential can be measured. It has been determined by various workers (7, 8, 9). Thermodynamically reversible potentials in the range pH 2 to pH 5.75 can be measured [Borsook & Keighley (9)]. Within these limits, the potential is proportional to the ratio of the concentrations of the reduced and oxidised forms, and the observed and calculated values agree; the potentials vary between —281 and —104 mv.

The kinetics of the reduction of cystine and related dithio-acids by reversible inorganic oxidation-reduction systems of strong reducing power have engaged the attention of Preisler (10). His results are of considerable interest with regard to glutathione.

Of greatest interest are the oxidation-reduction potentials of biological oxidation catalysts and systems closely related. Using the crystallised material, Conant & Pappenheimer (11) have recently measured the oxidation-reduction potential of the haemoglobin-methaemoglobin system. They find $E_0 = 152 \pm 5$ mv. at pH 7.0 and $\mu = 0.3$. Using either the protein-free material in solution or a suspension of yeast cells which had been plasmolysed with NaCl in phosphate buffer, Coolidge (12) has obtained, for Keilin's cytochrome, an oxidation-reduction potential of +260 mv., which is independent of the oxygen pressure. According to him only 0.3 per cent of the iron in yeast is probably present in the cytochrome. The reversible oxidation-reduction potential of the purple-blue pigment of the nudibranch, *Chromodoris zebra*, which is possibly a respiratory pigment, has been found by Preisler (13) to be $E_0 = -102$ mv. at pH 7.0.

A further reversible equilibrium employing a dehydrogenase (14) has been associated with the reversible equilibrium, succinic acid–succinodehydrogenase–fumaric acid, which was studied potentiometrically with the help of oxidation-reduction indicators by Lehmann, Thunberg, and Quastel & Whetham. Lactic acid is oxidised reversibly by the dehydrogenase of heart muscle acting in conjunction with a coferment. Lactic acid, pyruvic acid, the dehydrogenase and the coferment form a thermodynamically reversible oxidation system in the presence of janus green or neutral red, whose potentiometrically measured value is $E_0 = -181$ mv. The oxidation of β-hydroxybutyric acid to β-ketobutyric acid, which is brought about by the same enzyme, forms also a reversible system. The free energy of the lactic

acid oxidation agrees very well with the value calculated from thermal data.

Szent-Györgyi (15) suggests the use of an oxygen electrode in place of the hydrogen electrode, since, on the one hand, all biological oxidation-reduction potentials will then have the same sign, and, on the other hand, the cell creates its energy from the potential difference reckoned with regard to this electrode.

The Dehydrogenases and Their Reactions

According to Wieland's theory, the dehydrogenases are those enzymes which are responsible for the activation of hydrogen and its transportation within the cell. Our conception of their action has lately been somewhat modified. As well as acting specifically toward hydrogen donators they possess a specificity for hydrogen acceptors (16). They are not able to use any hydrogen acceptor thermodynamically possible. It is therefore convenient to divide the dehydrogenases into the oxytropic, i.e. those which use oxygen as acceptor, and anoxytropic, i.e. those which can bring about definitely anaërobic dehydrogenation (dismutation) reactions. As a result of this doubly specific affinity, a ternary complex may be held responsible for the functioning of dehydrogenases, namely donator-enzyme-acceptor (16). Many dehydrogenases exhibit a decidedly specific relationship toward only one donator. Succinodehydrogenase, for example, is able to use only monomethylsuccinic acid besides succinic acid itself (17). The heart-muscle dehydrogenase already mentioned is also very specific together with its coferment (14). Apart from lactic acid it is able to attack only β-hydroxybutyric acid and fructose diphosphate. This specific activity is sometimes interconnected with steric conditions. Malicodehydrogenase from seeds can only dehydrogenate *l*-malic acid without acting upon the *d*-form at all (17). Thunberg (17) believes that the specific activity lies partly in their ability to fix certain substances to themselves and partly in the activation of the hydrogen of such substances.

Bacterial dehydrogenases.—Quastel (18) has recently summarised work done in this field. Without doubt the dehydrogenating ferment system of acetic bacteria has been investigated the most intensively. As is known, acetic fermentation was the first biological process on which the validity of the dehydrogenation theory was tested (19). Wieland & Bertho (20) have thoroughly investigated this process in order to settle general questions concerning the dehydrogenation

theory. It has been shown that it consists of a series of dehydrogenations with aldehyde as an intermediate product. The three following reactions take place: Alcohol → acetaldehyde (I); acetaldehyde (hydrate) → acetic acid (II); 2 acetaldehyde → acetic acid + alcohol (III). Aldehyde can therefore be converted to acetic acid in two ways. Wieland & Bertho have found the third process to be of only secondary importance in acetic fermentation. According to a recent publication of Windisch (21), however, the formation of alcohol (process III) should be given an important position in aërobiosis to agree with Neuberg's ideas (22). The production of nascent intermediate substances in cell metabolism should bring about an acceleration of the reaction so that from the results of the reaction nothing can be concluded about its course. Wieland & Bertho attribute processes I and II to the action of a dehydrogenase. It is questionable whether process III can be due to the same ferment, acting then, of course, as a mutase.

Müller (23) has investigated the action of dead bacteria on isopropyl alcohol using oxygen and quinone as acceptors. Since the ratio of oxygen activity to that of quinone [25 to 30:100, about the same as with live acetic bacteria (20)] was not affected by such selected preliminary treatment as washing, etc., clearly only one alcohol-dehydrogenase—in Wieland's sense—comes into consideration. It is destroyed at a temperature of 54.5° C. Therefore oxygen cannot possibly be activated by cytochrome or genuine oxidases. Hydrocyanic acid has a much greater detrimental effect on the oxygen respiration of dead acetic bacteria than on the quinone respiration, but is not nearly so reactive to dead as to living bacteria. Müller (24) has also studied the oxidative decomposition of methyl alcohol by live and dead acetic bacteria. Living *B. pasteurianum* can almost completely oxidise it while acetone bacteria only oxidise it to the aldehyde stage. He asserts that three enzymes act in this case, namely an alcohol dehydrogenase, and aldehydrase and an enzyme capable of decomposing formic acid, which is very sensitive to treatment with acetone.

The oxidative decomposition of glucose to carbon dioxide and water by such lactic-acid-forming bacteria as *B. Delbrückii* and *acidophilus* is known to be a dehydrogenation process (25). The oxygen used appears as hydrogen peroxide since the bacteria are free from catalase. The respiratory quotient is therefore 0.5. *B. bulgaricus* [Fromageot (26)] as well as the pneumococci closely connected to the lactic-acid-forming bacteria act very similarly (27). Davis (28)

has confirmed the foregoing with regard to *B. Delbrückii* and has also observed that lactate is oxidised, for the oxidation of lactate is not prevented by iodoacetic acid, while that of glucose is. Lineweaver (29) has demonstrated the presence of typical dehydrogenases in *Azotobacter vinelandii* with the help of the methylene blue technique.

Schott & Borsook (30) have shown that coupled reactions in biological systems require the help of auxiliary substances. For instance the presence of methyl violet is necessary for the reduction of pyruvate to lactate using *B. coli* treated with toluene as the ferment system. This reduction is brought about by the energy set free by the anaërobic oxidation of formate to bicarbonate. Again methylene blue is necessary in the case of the reduction of fumarate to succinate which is brought about by the energy of the anaërobic oxidation of lactate to pyruvate.

The dehydrogenating enzymes of yeast.—Nothing is yet known about the different dehydrogenases in yeast and the part which each plays in the various dehydrogenation processes. According to Euler (31, 32) the so-called carbohydrate redoxase or hexosephosphate dehydrogenase, which is an oxidation-reduction system activated by cozymase, occupies an important position in the decomposition of carbohydrate by yeast. As a matter of fact, adenylpyrophosphoric acid is quite as good a coferment as cozymase in the dehydrogenation of hexosephosphates by extracts of plant seeds (33). It may be assumed that this dehydrogenase system dehydrogenates the substances containing three carbon atoms resulting from glycolysis. It has been observed that the decolourisation of methylene blue in pure alkaline sugar solution results in the intermediate formation of dihydroxyacetone and reductone (enoltartronaldehyde). From this Euler (34) presumes that the enzymatic decolourisation of methylene blue, by dehydrogenases in co-operation with cozymase, gives as intermediate products dihydroxyacetone or a reductone. On the other hand certain results point to a direct attack on the sugar molecule. In the animal organism such dehydrogenases, as glucose dehydrogenase (35), are known, which are able to attack sugar directly. Gluconic acid is probably the only oxidation product (36). Harrison maintains that the activity of glucose dehydrogenase is connected with the presence of an aldehyde group (37).

Yeast, "impoverished" of its reserve materials by shaking for twenty hours with oxygen, has definite advantages for the investigation of its dehydrogenating mechanisms (38). Yeast is able to de-

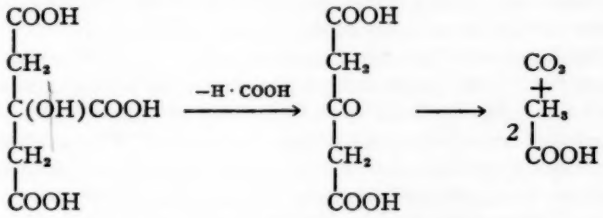
hydrogenate alcohol to acetic acid in presence of oxygen or methylene blue. It has been found that after one hour's treatment with oxygen half of the alcohol used up has been oxidised to acetic acid while the other half has gone to carbon dioxide. The R.Q. is therefore 0.5 (39). Succinic acid is also formed, equal in amount to the sum of the acetic acid and carbon dioxide formed, subtracted from the amount of alcohol used up. Aldehyde can also be dehydrogenated but when high concentrations are used dismutation takes place to a greater extent than dehydrogenation (38, 40). The close relationship between biological oxidation and anaërobic fermentation is clearly shown in this simple case. Lactic acid, too, can be dehydrogenated by bottom yeast to acetic acid, showing the important position occupied by the latter in oxidative metabolism. Using lactic acid dehydrogenase from fresh yeast, the decrease of lactic acid and the formation of pyruvic acid can be demonstrated (41). Since methylglyoxal is dehydrogenated considerably faster than lactic acid, the latter is probably not an intermediate product, according to Wieland & Claren (38). Acetic acid can be dehydrogenated by yeast but only in the presence of oxygen (42). Water and carbon dioxide are the products. If the oxidation is stopped after it has gone a considerable length, 5 per cent succinic acid and 10 per cent citric acid (as lead salt) are obtained. Ethyl alcohol is not formed. The succinic acid clearly results from the removal of hydrogen from the methyl groups of two molecules of acetic acid as first suggested by Thunberg.

$$2\,HOOC \cdot CH_3 - 2\,H \rightarrow HOOC \cdot CH_2 \cdot CH_2 \cdot COOH.$$

Yeast acts on succinic acid only one-third as fast as on acetic acid, but on the surface of the enzyme succinic acid is formed from acetic acid in an activated state and possesses a heat of activation only a small portion of which is given up, resulting in the formation of only a certain amount of normal molecular succinic acid. This explains how this stage in the oxidation can be passed.

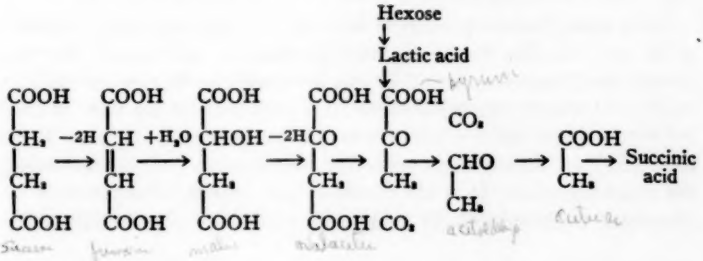
The formation of citric acid can be attributed to the aldol condensation of oxalacetic acid and acetic acid, but all attempts to bring such a condensation about have been futile. The carbohydrate metabolism of yeast is clearly analogous to that of moulds and fungi. The enzymatic decomposition of sugar into alcohol and carbon dioxide always precedes the synthesis of succinic acid. Since, as will be mentioned in the next section, the conversion of acetic acid to succinic acid represents the only stage lacking up till now in the oxidative degradation

which takes place in muscle, the reaction order in the case of yeast appears to be established and is as follows: Carbohydrate → methylglyoxal or lactic acid → pyruvic acid → acetaldehyde and carbon dioxide → acetic acid → succinic acid → fumaric acid → malic acid → oxalacetic acid → pyruvic acid and carbon dioxide, etc. This takes place only when oxygen and not methylene blue is used. So far malic acid has not been found, but very recently fumaric acid has been isolated as a product of the oxidation of alcohol (Wieland, unpublished). Wieland & Sonderhoff (43) have studied in this connection the anaërobic fermentation of citric acid by yeast. Acetic acid, formic acid, carbon dioxide, and succinic acid are formed. The acetic and succinic acids can alone be oxidised further. This means that fermentation is the first step in the enzymatic oxidation of citric acid. As shown in the following scheme, the yeast must contain a ferment which can split off formic acid:



These reactions must be accompanied or followed by dehydrogenation, thus explaining the formation of succinic acid. Recent observations, as yet unpublished, have shown that free hydrogen is liberated.

Muscle dehydrogenases.—As a result, especially of the work of Hahn (44), the reaction order mentioned above, with the exception of the stage acetic acid to succinic acid, has been definitely established.



Muscle succinodehydrogenase converts succinic acid into fumaric acid, which is transformed further into malic acid by the action of a hydratase. Malic acid can be degraded to pyruvic acid, by way of oxalacetic acid, by oxygen as well as by methylene blue, but only when the heart muscles of a freshly killed animal are used. The particular ferments which take part in this conversion are not known one from another, because, as soon as the cell material is dead, malic acid can no longer be transformed.

Muscles possess a specific lactic acid dehydrogenase (14, 41) which causes a reversible oxidation-reduction system to come into play. Pyruvic acid is formed, which, according to Hahn, is always present in the cell. This provides the connection with the anaërobic part of carbohydrate metabolism. That the reaction order is actually as given above and can also proceed in the reverse direction to reach a certain equilibrium has been supported by Stöhr's results (45); four hours after male rats had been fed on succinic acid, the glycogen content of their livers increased very considerably.

The following other dehydrogenases occurring in the animal organism have been mentioned: a hydroquinone dehydrogenase in muscle (46), the so-called liver enzyme which brings about the dehydrogenation and disproportionation of aldehyde (47), a series of anoxytropic dehydrogenases similar to the hexosediphosphate dehydrogenase from acetone-liver and muscle (48), the citricodehydrogenase (49) and the glucose dehydrogenase from liver (35), and finally a ferment present in the pancreas which can dehydrogenate fat (50). According to Bernheim (51) proline and hydroxyproline can be dehydrogenated by ground liver. The nature of the dehydrogenation product is unknown. While urethane generally inhibits dehydrogenase action, this one is specifically connected with veratrine (52). The same applies for the dehydrogenation of xanthine by liver.

Coferments and Sulphydryl Compounds

In carbohydrate degradation, both aërobic and anaërobic, adenylic acids, particularly, come into the question as activators. As has already been mentioned, and as can be shown to be true in isolated cases, it is clearly the dehydrogenation processes taking part in carbohydrate degradation which require such coferments. Euler & Myrbäck (53) are mainly responsible for the investigations on cozymase, the yeast activator. It is not identical with muscle adenylic acid or adenosine triphosphate. It is a mononucleotide which analytically

closely corresponds to muscle adenylic acid and has not yet been crystallised (54). Cozymase preparations lose their activity completely when acted upon by animal phosphatase, since all the phosphoric acid is split off; this gives further support to the belief that the nucleotide isolated from yeast is identical with cozymase (55). Cozymase acts as coferment to the carbohydrate redoxase, according to Euler (31, 32). It can be shown that the enzymatic dehydrogenations of malic acid, lactic acid, citric acid, ethyl alcohol, and glutamic acid using methylene blue are activated by a cozymase preparation from boiled yeast juice (56). The coferment of lactic acid oxidation by the dehydrogenase of heart muscle is a monophosphoric acid monoadenylnucleotide which is not identical with muscle adenylic acid nor with adenylpyrophosphate but possibly with cozymase (57). Adenylpyrophosphoric acid has a powerful activating effect on methylene-blue reduction by means of muscle dehydrogenase. This is no doubt due to an activation of the dehydrogenation (58). The nucleotide isolated by Embden from heart muscle is just as able as adenosine triphosphoric acid to accelerate methylene-blue decolourisation in the presence of plant and animal dehydrogenases. Probably in these two cases the same dehydrogenase is being activated (59). Holmberg (60) believes that the activation of methylene-blue decolourisation by certain plant seeds, using cozymase and adenosine triphosphate, is brought about by these substances acting as donators.

Lohmann (61) and Barrenscheen *et al.* (62) have put forward suggestions as to the constitution of adenylphosphoric acid. Lohmann supposes that the two easily hydrolysable phosphoric acid molecules are not connected to the amino groups of the adenine, while Barrenscheen, on the other hand, believes that they are bound like an iminopyrophosphoric acid.

The rôle of the sulphydryl bodies, especially glutathione, in cell metabolism is not yet definitely known. They certainly serve at least to regulate the relationships of the potentials in the various cell reactions (*vide supra*). The significance of glutathione as coferment for methylglyoxalase is quite definite [Lohmann (63)]. The supposition that the thiosystems act as carrier catalysts by virtue of oxidative splitting is faced with the difficulty that a very strong negative reduction potential is necessary for the conversion of SS- to SH-compounds (10).

Waldschmidt-Leitz *et al.* (64) have put forward a hypothesis concerning the central position of the sulphydryl compounds in the regu-

lating of the intracellular transformations of protein and carbohydrate and their connection with the oxidation-reduction processes. The addition of iodoacetic acid would stop the functioning of the sulphydryl in enzyme reactions by oxidation to the SS-form, since these reactions depend on the presence of sulphydryl metal complexes. A test for the influence of sulphydryl on enzymatic reactions could be formed on this basis. The cessation of lactic acid formation in muscles poisoned with iodoacetic acid, in which case, as opposed to Lohmann (65), the presence of sulphydryl should be necessary, would be so explained. Sulphydryl is also necessary in yeast fermentation (66). The excessive lactic acid fermentation which takes place in malignant tumors can be put down to the decrease in oxygen potential due to sulphydryl. Bierich & Rosenbohm (67) could detect no glutathione in nearly all normal body tissues with the exception of blood, which means the abandonment of the idea that glutathione takes part in reversible oxidation-reduction processes.

Schöberl (68) and Pirie (69) have carried out model experiments on the oxidative degradation of sulphydryl compounds.

Warburg's Ideas with Regard to Cell Respiration

Warburg's *Atmungsferment* is an unspecific oxygen carrier whose active group is a haemin closely related to the phaeohaemins. Although, as Wieland's theory postulates, through its supposition of the existence of dehydrogenases, a certain selection of the substrate to be oxidised must take place, nevertheless Warburg believes that the combustible materials in the cell must be prepared for the combustion. He calls this process, which determines the extent of oxygen respiration, activation of the substrate (70). An enzyme system capable of activating hexosemonophosphate (Robison's ester) is present in blood corpuscles. It consists of enzyme and coenzyme, which can be obtained separately from the blood corpuscles of the rat or from yeast, and from the blood corpuscles of the horse respectively (71).

The following scheme gives the mechanism of the action of this enzyme system in the activation of substrates:

System	Oxidised by
Hexosephosphate + Enzyme + Coenzyme	Oxygen
	Methylene blue/leuco methylene blue
	Methylene blue/leuco methylene blue + oxygen
	Haemoglobin/methaemoglobin + oxygen
	Haemoglobin/methaemoglobin + methylene blue/leuco methylene blue + oxygen

According to this scheme the activated hexosephosphoric acid can be oxidised by oxygen or methylene blue or by oxygen with methylene blue as carrier. Then again, the system haemoglobin/methaemoglobin can also be used as catalyst. When it reacts directly with oxygen it takes over the rôle of *Atmungsferment*, for the fact that methylene blue can react directly or through haemin iron with the activated hydrogen holds good also for the cell itself (72). Only the last process is sensitive to hydrocyanic acid as well as to carbon monoxide. On the other hand Wendel (73) comes to the conclusion, from his studies in the oxidation of lactic acid by erythrocytes from dogs in the presence of methylene blue or oxygen, that methaemoglobin is not essential for the oxidation in this system; hydrocyanic acid accelerates the reaction. This acceleration is probably not a part of the oxygen-activating mechanism, but may be traced to a combination of the hydrocyanic acid with pyruvic acid.

Warburg believes that he has proved the existence of the *Atmungsferment* in yeast and acetic bacteria by direct spectroscopic methods (74, 75).

Warburg & Christian (76, 77) have isolated a further type of oxidation-ferment from bottom yeast and lactic-acid bacteria. It is free from haemin bodies, and is characterised by its absorption spectrum. Lactic-acid bacteria contain 77 mg. per kilo, while yeast contains 20 mg. per kilo. This new oxidation-ferment acts as a carrier catalyst by taking up the activated hydrogen from the substrate, going, itself, from the yellowish-red to the colourless leuco form, which in turn is oxidised by oxygen giving rise to hydrogen peroxide (77). That hydrogen peroxide is formed when glucose is oxidised by lactic-acid bacteria has been established by Bertho & Glück (25). This has been interpreted as a typical dehydrogenation process. Warburg & Christian (78) believe that they have disproved Wieland's theory by this new discovery, but that is not so, for the intermediary action of the yellowish-red ferment does not do away with the reality of dehydrogenation. In cells which contain the *Atmungsferment*, the oxidation of the leuco form is brought about by oxygen activated by phaeohaemin or possibly by the intervention of biological ferro–ferri systems (cytochrome) (79). The new yellow-red ferment belongs to the class of flavines or lyochromes, colouring matters widely distributed in the animal and plant kingdoms. It can be split into a "carrier" of high molecular weight, and the real colouring matter, which is closely related to ovoflavine and lactoflavine (80, 81). It is known

that both of these crystalline flavines can act as hydrogen acceptors, and are able to dehydrogenate biological substrates [Wagner-Jauregg & Ruska (82)]. They have also biological oxygen-carrying properties. Vitamin B_2 is very closely connected to the flavines and, indeed, may possibly take part in the oxidation processes in the cell. The active group of the yellow-red ferment is a colouring matter easily soluble in water and possessing a green fluorescence. Its composition is $C_{13}H_{18}N_4O_2$, and it melts at 329° (80).

The transformation product obtained by exposing to light in *N/2* NaOH solution gives on hydrolysis 0.73 mol of urea per mol of substance hydrolysed (81).

Svedberg & Eriksson (83) have determined the molecular weight of the new oxidation ferment by ultracentrifuging and have obtained values between 50,000 and 70,000. The nitrogen content of 2.9 per cent and the specific volume of 0.655 do not indicate the presence of protein in the colloidal "carrier."

Catalase, Peroxidase, and Oxidases

Catalase was the first oxidation-ferment in which a haemin complex was shown to be the active group by direct spectroscopic investigation of its highly purified solution [Zeile & Hellström (84)]. Kuhn, Hand & Florkin (85) obtained analogous results with peroxidase. In both cases the haemin is very probably identical with protohaemin, and the iron is probably trivalent. HCN inhibits both ferments considerably, while CO does, if at all, only very slightly (85*a*).

The absolute activities of peroxidase, catalase, and Warburg's *Atmungsferment* are of approximately the same order (86). One mol of these three ferments can convert about 10^5 mols of H_2O_2 or oxygen respectively. On the one side the specific nature of the iron linkage in the haemins and, on the other side, the way in which the haemin-like active group is adsorbed determines the exceptionally high activity of these haemin-containing ferments. The influence of these factors is given in the following table:

1 mol (gram-atom) catalase-iron decomposes 6×10^4 to 2×10^5 mol H_2O_2 at 0°
1 mol (gram-atom) haemin decomposes 0.01 mol H_2O_2 at 0°
1 gram-atom Fe^{++} or Fe^{+++} decomposes 0.00001 mol H_2O_2 at 0°

Reuter, Willstaedt & Zirm (87), who have recently carried out experiments with different iron complexes in order to find out how the iron in peroxidase is combined, have come to the conclusion that

the porphyrin belongs to the haemin type and not to the chlorophyll. Stern (88) has reported on the catalytic activity of haemin complexes. In no single case has the activity of the haemin been raised to the same order as that of catalase by complex formation.

Noteworthy is the discovery of Malkov (89) that the activity of yeast catalase and peroxidase, like oxidation processes in general, is inhibited by phosphate, probably as a result of partial fixation of the iron constituent of the ferment.

Albers (90) has reported on intermediate products formed during the catalytic decomposition of hydrogen peroxide. He finds that the hydrogen peroxide forms two kinds of compounds with the catalase molecule: a reactive catalase — $(H_2O_2)_2$, and an unreactive catalase — $(H_2O_2)_6$. This agrees with Wieland's conception of catalase as a dehydrogenase with specific hydrogen acceptor.

Schwab, Rosenfeld & Rudolph (91), on the other hand, have interested themselves in the chain nature of the catalase action. According to the Haber-Willstätter theory, as is well known, the radicle causing chain formation in hydrogen-peroxide decomposition by catalase and in autoxidation is the same, namely OH. It is to be expected that the unspecific action of this chain-reaction catalyst would result in a similar behaviour of similar reactions toward the same inhibiting substances. But such a comparison has shown that the inhibiting substances do not act on an identical chain reaction. One is therefore forced to conclude that the course of chain reactions is specific and conditioned by the mechanism of their uncoupling. This is also of importance in enzyme reactions (92).

Guthrie (93) has put forward an iodometric method for the estimation of the action of potato-oxidase. Szent-Györgyi & Victorisz (94) have shown that the action of potato-oxidase is increased at least fifteen times by hexuronic acid. The complex enzyme system established by Barron & Hastings (95) in gonococci may be an α-hydroxidase containing a heavy metal. It oxidises α-hydroxy acids to α-keto acids. The system consists of two components—a coenzyme sensitive to hydrocyanic acid, which activates the substrate, and another hydrocyanic-acid-sensitive enzyme, which acts as oxygen carrier. Kisch & Schuwirth (96) have put forward their model researches on quinone as an example of enzyme action, and reported on the catalytic oxidative deaminisation of amino acids in the presence of oxygen and hydrogen acceptors.

Franke (97) has studied the action of carotenoids in the autoxida-

tion of unsaturated fatty acids. They act as positive catalysts only on the free fatty acids and not on triglycerides, presumably by uncoupling of the reactive chain as a result of easier ability to autoxidise. In this connection vitamin A and carotene occupy no special position. An increase in oxidation is obtained by the addition of haemin probably due to the formation of an addition compound of haemin and carotenoid.

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ENZYMES*

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Considerable progress has been made in enzyme research during the last two years. Among the studies published during that time dealing with the mode of action of the enzymes in general, experiments on enzyme models are particularly worthy of note. They aim at interpreting the catalytic activity of the enzyme on a chemical basis. Thus Bredig & Gerstner (1) have shown that cotton, when a dimethylamine group is added to it, acquires catalytic properties, e.g., the property of splitting carbonic acid from β-keto-carbonic acids, or the property of forming optically active mandelic-acid nitrile. It is, therefore, an enzyme model of known composition, consisting of a high molecular colloidal bearer and a specific active group linked to it, corresponding to the theories of the nature of the enzymes as developed by Willstätter (2). This particular theory has been strikingly supplemented by Langenbeck's experiments (3), also carried out on enzyme models. His experiments lead to the assumption of special "activating" groups in the enzymes existing in connection with the active groups proper, thus permitting of an explanation for the high catalytic activity of the enzymes. The extraordinary increase in the activity of the amines in splitting keto-acids by introducing certain activating substituents into the amine may be quoted as an example. The problem of the nature of the compounds formed by the reaction between enzyme and substrate has also been further pursued, particularly the phenomenon of inhibited reaction when the substrate concentration is high. This has been explained in the case of catalase by assuming an enzyme-substrate complex, which cannot be further decomposed [Albers (4)], and in the case of ester-splitting as the consequence of a displacement of water on the surface of the enzyme [Fischgold & Ammon (5)].

Esterases.—Researches undertaken in the field of ester-splitting enzymes are mostly occupied with their specificity, for instance, their capacity for optical selectivity. In the case of esterase from human liver, steric specificity is found to be independent of its stage of growth; it is constant also for all animal species [Bamann,

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Mahdihassan & Laeverenz (6)]. Two different active groups in the enzyme are held responsible for the liberation of both optic antipodes in mandelic-acid esters, one reacting with the laevorotatory and the other with the dextrorotatory ester; but it is to be assumed, not that two absolutely specific enzymes are present together, but that one and the same active group has varying effects in its field of action according to its linkage with the colloidal bearer of the enzyme [Schwab, Bamann & Laeverenz (7)]. Further examples have been given [Ammon & Geisler (8)] of the shifting or even reversal of configurational specificity, depending on the concentration of the substrate. According to Rona, Ammon & Trurnit (9), on the other hand, an asymmetrical synthesis can be effected by esterase from optically active alcohols only, and not from optically active acids. The position of the equilibrium, reached from both sides, between the synthesis and the hydrolysis of the ester seems to undergo a shift when other materials, e.g., calcium salts, are added [Rona & Ammon (10)].

Stedman, Stedman & Easson (11) have described in serum from horses, along with the usual esterase, an enzyme which is apparently specific for the splitting of the choline esters, acetylcholine or butyrylcholine.

Virtanen & Suomalainen (12) have observed an interesting transformation of exogenous lipase in the animal organism. They report that, when lipase from the pancreas of a pig was intravenously injected into rabbits, an approximately corresponding increase in liver-esterase content occurred. The lipase thus stored up in the liver is, however, true liver esterase; it acts, for example, very slightly on olive oil and is not inhibited by atoxyl; enzymatic specificity in the organism, therefore, adapts itself to the environment.

Phosphatases, sulfatases, tannase.—In the field of the specificity of phosphatases, numerous fresh facts have been established and familiar ones elaborated. The kinetics of glycero-phosphate cleavage by kidney phosphatase has been examined by Jacobsen (13). The determining factors, above all, are the inhibitory action of the liberated phosphate and the concentration of the substrate. Only in a few cases is the reaction equation of the first order. According to Roche (14) we must differentiate two classes of phosphatases in the animal organism, that of the red and that of the white blood corpuscles, to the latter of which those of all other organs belong; they are characterised by a different pH optimum (6.0–6.2 or 8.8–9.0). Besides

these phospho-mono-esterases it would appear as if we must also assume a pyrophosphatase [Jacobsen (15), Kurata (16)], accompanied by a specific activator, and also a special phospho-di-esterase, the separation of which from mono-esterase is described in an example by Uzawa (17), but its existence still seems doubtful. Certain it is that besides phospho-esterases, special phospho-amidases exist [Waldschmidt-Leitz & Köhler (18); Ichihara (19)], which, for instance, accomplish the cleavage of guanidine-phosphoric acids, or of the phosphoric acid amides; their separation by adsorption methods has also been accomplished.

The hydrolytic action of phospho-esterase from kidney and from tumours has been shown to be specifically inhibited by sulfhydryl compounds; disulfide compounds were found to have no effect thereon [Waldschmidt-Leitz & Schäffner (20); Edlbacher & Kutscher (21)]. This phenomenon, which Edlbacher & Kutscher (21) seek to explain by assuming that heavy metals are involved in the active group of the enzyme, is found only in alkaline zones [Lohmann (22)]; its physiological importance is therefore not yet clear. Contrary to phospho-mono-esterase, liver adenosine-triphosphatase, which has been distinguished from it, is activated by sulfhydryl, but inhibited by magnesium ions [Barrenscheen & Lang (23)].

While the nucleotidase of the intestinal mucosa can be regarded as identical with ordinary phospho-mono-esterase [Levene & Dillon (24)], we must differentiate in the digestive tract two separate enzymes both of which play a part in breaking down nucleic acids: on the one hand a poly-nucleotidase [Levene & Dillon (25)] or nucleinase [Klein (26)], which decomposes the nucleic acids into nucleotides, and on the other, a mono-nucleotidase or nucleo-phosphatase [Klein (27)]. It has been found possible to eliminate the action of the nucleo-phosphatase in the mixture of the two enzymes by inhibition through arsenate, for example, which has served in the preparation of nucleotides.

Specificity researches have also been carried out with regard to two particular groups of esterases, viz., sulfatases and tannase. Glucose- and saccharose sulfuric-acid esters are decomposed, as Tankó (28) has shown, by chondro-sulfatase found in bacteria [Neuberg & Hofmann (29)], but not by kidney sulfatase; the former has therefore a more extensive specificity. A preparative partial separation of the component enzymes of the myrosinase complex into sulfatase and thio-glucosidase has been described [Neuberg & Schoenebeck (30)].

Noteworthy results have been obtained concerning the specificity of tannase, which permit of the unmistakable differentiation of this enzyme from ordinary mold esterase [Dyckerhoff & Armbruster (31)]; tannase solutions free from esterase were obtained by selective destruction in slightly alkaline medium of the esterase in mold extracts. In contrast to esterase, tannase splits only those esters whose acid component contains at least two phenolic hydroxyl groups in the molecule, neither of which, however, can be ortho to the carboxyl group; the alcohol component, on the other hand, is found to be of no importance for the specificity. Moreover, a direct binding of the ester-carboxyl to the oxidised benzene ring is essential for the cleavability of an ester by tannase.

Proteinases.—Investigation into the chemical nature of pepsin has been furthered by a discussion of the uniformity of the preparations obtained by Northrop (32) in the form of crystalline proteins. The objections, supported by experiments [Waldschmidt-Leitz & Kofrányi (33); Dyckerhoff & Tewes (34)], that it is possible to adsorb on to crystalline plant globulin the active part of pepsin from solutions of crystalline pepsin, the pepsin-protein being left inactive, have been repudiated by Northrop (35) on the grounds that this was really an adsorption of the entire pepsin-protein molecule to plant protein; the claim, therefore, that crystalline pepsin represents the pure enzyme is still maintained. On the other hand Willstätter & Rohdewald (36) point out that it is possible to obtain pepsin preparations free from protein by other means. Part of the pepsin is to be found, it is true, in the mucous membrane of the stomach in insoluble form, probably linked to protein, as desmo-pepsin. The presence in the mucous membrane of an inactive pro-pepsin, a preparatory stage of the enzyme, has also been described [Ege & Menck-Thygesen (37)]. It undergoes activation by hydrogen ions at a velocity measurable at pH 5, but too high to be measured at pH 3.

Other investigations are concerned with the products of the peptic cleavage of protein. Thus the peculiarity of the peptic digestion of casein, which is characterised by a preliminary increase in viscosity, is explained by the formation of an intermediary product rich in phosphorus and difficultly soluble [Holter, Linderström-Lang & Funder (38)]. The mitogenetic analysis of pepsin action [Billig, Kannegiesser & Solowjew (39)] as well as its analysis by the Tyndall phenomenon show the breaking up of peptide linkages by the enzyme.

The differentiation of the protein-splitting pepsin from rennin, which only coagulates milk, has made further progress. Tauber & Kleiner (41) report on the preparation of rennin free from pepsin procured by alcohol precipitation of extracts from the calf stomach; rennin also occurs here in the form of an inactive preparatory stage, but this is converted into active enzyme by a smaller concentration of hydrogen ions than is the pro-pepsin. The nature of the action of rennin cannot yet be more exactly characterised [Holter (42)], but it is certainly based on a chemical change in the casein.

The description of lyo- and desmo- forms of the enzymes, i.e., of soluble forms and of forms linked to insoluble cell constituents, has been extended to trypsin [Willstätter & Rohdewald (43)]. Thus in the white blood corpuscles and in the pancreas two forms of trypsin have been differentiated, one soluble in glycerine and the other not; pancreatic desmo-trypsin cannot be activated further by enterokinase. A comparison between these lyo- and desmo-forms seems pertinent to the question of the protein nature of enzymes. Experience at present indicates that the enzymatically active groups are linked to an alterable colloidal bearer which can also be broken down, and that we must further distinguish loosely associated concomitant materials—the "tugs" of the enzyme. Northrop & Kunitz (44) have described a crystalline trypsin preparation of the nature of protein and of high and constant activity procured by fractional salting out with ammonium sulfate; its molecular weight is given as 34,000. The preparation coagulates blood, but milk only to a small degree, and it cannot be further activated by enterokinase. The extent of its action on proteins is less than that of cruder trypsin preparates. When the enzyme solutions are heated for a rather long period of time and also when pepsin or alkali is added, the loss of activity is parallel to the change in the protein. It is remarkable that no loss of activity and no denaturation is observed when the solution is heated for a short time to 100° and quickly cooled again. From these results the investigators conclude that the tryptic activity of the preparation is a property of the native protein molecule.

In the case of the animal intracellular proteinase, cathepsin, we must also distinguish a desmo- and a lyo-form [Willstätter & Rohdewald (36)]. In this connection Hedin (45) describes a particular enzyme in the spleen which synthesises protein and which can be separated from the so-called α-protease by means of fractional extraction. By use of the ultracentrifuge, Svedberg & Eriksson (46)

have studied the mechanism of the breakdown of protein in egg albumin by papain, the plant proteinase corresponding to cathepsin. The process seems to consist in the gradual diminution of the albumin molecule by the splitting off of fractions of low molecular weight. Experiments on the proteinases in the formed elements of blood [Willstätter, Bamann & Rohdewald (47)] have been continued by Kleinmann & Scharr (48). According to them, leucocytes contain trypsin and cathepsin, which can be separated, while lymphocytes contain only cathepsin. The further assumption, that cathepsin is only activated by sulfhydryl in the process of gelatine splitting, has since been refuted [Waldschmidt-Leitz, Scharikova & Schäffner (49)].

The activation of intracellular proteolysis by sulfhydryl compounds, and its possible connection with respiratory metabolism in the cells, has been most carefully and critically examined. In tumours, where respiration is reduced, less cathepsin is found than in other organs of carcinomatous animals [Maschmann & Helmert (50); Rondoni (51)]. The degree of activation of the enzyme is less there, as is also the content of reduced glutathione [Heinlein (52)]. According to this, there should be no simple connection between respiration and the transformation of protein in animal organs and in tumors. The assumption that the inhibitory action of iodoacetic acid on proteolysis in the organs is due merely to an elimination by oxidation of the sulfhydryl compounds [Michlin & Rubel (53)] has been corrected [Maschmann & Helmert (54)]; what takes place is an inhibition of the activated enzyme itself, by iodoacetate ions. According to Bersin & Logemann (55) the activation of papain by sulfhydryl compounds is to be interpreted as a reduction of the enzyme itself which is present in the oxidised form, containing disulfide groups; it occurs also under the influence of other reducing agents. Ascorbic acid (vitamin C) has also an activating effect in this sense on proteolysis by cathepsin, but not, strange to say, on papain [Karrer & Zehender (56); Purr (57)]. Another noteworthy point is the antagonistic influence of oxidising and reducing agents on the transformation of protein; oxidation seems to favour synthesis of protein, and reduction, hydrolysis; this is reported of protein metabolism in animals [Rondoni & Pozzi (58)] and particularly strikingly in plants [Mothes (59)]. Rondoni (60) has described and discussed the possible physiological significance of an activation of cathepsin by phosphatides (lecithin, cephalin). This is due to

unspecific adsorption and is to be distinguished from activation by sulfhydryl; the enzyme, when fully activated by sulfhydryl, can undergo this further activation by phosphatides.

Proteinases which appear to belong to no uniform type, but rather to mixed types, have been frequently observed in bacteria and also in insect-eating plants, in so far as these have been examined. Acidoproteolytes, the bacteria of acid milk coagulation, contain a proteinase, an intermediary between papainase and tryptase, with a reaction optimum in an approximately neutral medium; it is not activated by sulfhydryl [Gorini, Grassmann & Schleich (61)]. An interesting phenomenon is that the proteinase of *B. fluorescens liquefaciens,* when cultivated on gelatine, is secreted quantitatively into the nutrient medium, while the peptidases present remain in the cell [Virtanen & Tarnanen (62)]. In the secretion of the tentacle-gland tissue of two kinds of nepenthes the presence of two true proteinases in each has been established, belonging, according to their reaction optimum, to the trypsin and the cathepsin type, but lacking the corresponding activating mechanisms [Stern & Stern (63)]. The secretion of *Drosera rotundifolia,* on the other hand, contains a proteinase, the properties of which place it between pepsin and papain [Holter & Linderström-Lang (64)].

Thrombin, the enzyme of blood coagulation, which is particularly differentiated from other proteinases, has been characterised more precisely. According to Mellanby (65) it is a proteolytic enzyme the action of which is said to consist in splitting fibrinogen into fibrin and serum globulin. Fischer (66) has purified thrombin from muscle extract, using as a control a method of quantitative determination. The enzyme itself is held to be a lipoproteid containing calcium and consisting of a protein component and a prosthetic group, probably cephalin.

Peptidases.—Most of the investigations on peptide-splitting enzymes are concerned with questions of their occurrence and specificity. Le Breton & Mocoroa (67) have established the fact that pure pancreatic juice contains inactive trypsin along with active carboxy-polypeptidase but no other peptidases. In pure intestinal juice, on the other hand, only the ereptic peptidases are found beside enterokinase.

On the basis of their experience in purifying amino-polypeptidase of the intestinal mucous membrane, Balls & Köhler (68) come to the conclusion that the enzyme itself contains phosphorus which dur-

ing dialysis is inactivated and split off in the form of phosphate ion. The relative activity of the enzyme with regard to a number of tetrapeptides was found to be constant, also when partially inactivated. No indication, therefore, was observed of the existence of several amino-polypeptidases. The distribution of peptidase in the root and coleoptile of the sprouting malt grain has been measured by a new micro-analytic method [Linderström-Lang & Holter (69)]; the accumulation of enzymes in certain sections of the root seems to be related to definite processes of growth.

The existence of a special enzyme, a prolinase, which splits proline peptides, e.g., in the intestinal mucous membrane, has been confirmed anew by Grassmann, Schoenebeck & Auerbach (70). Moreover, proline is found in certain proteins like gelatine to be bound in peptide linkages, as must be concluded from the deficit in free amino groups on hydrolysis with erepsin [Bergmann, Zervas & Schleich (71)]; such linkages with tertiary nitrogen seem to be split by amino-polypeptidase.

Bergmann & Schleich (72) have reported on the detection of a new enzyme in the kidney, a dehydro-dipeptidase, which splits unsaturated dipeptides; it is also found in putrid pancreas. Like a true peptidase, it attacks the peptide linkage itself. It decomposes, for instance, glycyl-dehydro-phenylalanine into glycine, phenyl-pyruvic acid and ammonia; the occurrence of this specific enzyme makes it probable that unsaturated peptides appear in metabolism as intermediates.

Abderhalden and co-workers (73) have carried out a great number of experiments on the specific actions of individual peptidases. Among the many isolated facts described by them, we would draw particular attention to their conclusion that the cleavability of optically active peptides by carboxy-peptidase is determined by the configuration of the amino acid at the carboxyl end of the peptide, whereas their cleavability by amino-peptidase depends on the configuration of the amino acid at the amino end of the peptide.

Remarkable experiments on the enzymatic cleavability of diketopiperazine-carbonic acids have been made by Matsui (74) and Ishiyama (75). Diketopiperazine-carbonic acid and also the anhydrides of glycyl-aspartic acid, glycyl-glutamic acid and the like, proved, in contrast to the non-carboxylated diketopiperazines, to be enzymatically cleavable by carboxy-polypeptidase, as long as they contained a free carboxyl group; ereptic enzymes and pepsin showed no action.

The possibility of the occurrence of diketopiperazine rings in proteins thus becomes once more a matter for discussion.

Aminoacylases

a) *Arginase.*—Researches carried out on this enzyme are mainly concerned with the continued study of its activation by sulfhydryl compounds [Salaskin & Solowjew (76); Waldschmidt-Leitz, Schäffner & Kocholaty (77)]. Contrary to the statements that sulfhydryl compounds like cysteine or reduced glutathione generally inhibit arginase action instead of activating it [Edlbacher, Kraus & Walter (78); Klein & Ziese (79)], it appears to be definitely established that not sulfhydryl alone, but heavy metals besides, play an important part in the activation of arginase [Salaskin & Solowjew (80); Waldschmidt-Leitz, Scharikova & Schäffner (49)]. The activating influence of sulfhydryl compounds is attributed by Edlbacher, Kraus & Leuthardt (81) to the fact that they take up oxygen, oxygen being an inactivator of arginase. Waldschmidt-Leitz & Kocholaty (82), on the other hand, have shown that activation takes place only in the presence of labile oxidation-reduction systems, independent, within certain limits, of their composition, while stable, oxidised, or reduced systems have either no effect or reduce activity. The mechanism of the activation is interpreted as a transmission of energy by an oxidation-reduction process taking place in the presence of the enzyme; the active form of arginase (non-saturated, in a peroxide form, or as free radical) is regarded as higher in energy content.

b) *Urease.*—The discussion on the homogeneity of crystalline urease has been continued. While Sumner & Kirk (83) point out that, in digestion with trypsin, loss of urease activity runs parallel to the breakdown of protein, Waldschmidt-Leitz & Steigerwaldt (84) point out that, if purified trypsin is used, the protein of the urease is indeed broken down but urease activity remains. Sumner, Kirk & Howell (85), on the other hand, draw conclusions as to the protein character of the enzyme from the fact that urease is completely inactivated by pepsin or activated papain. It seems that it is the extent and kind of proteolytic decomposition and not the decomposition as such which determines whether inactivation takes place or not.

c) *Desamidases.*—Guanase, an enzyme specific for desamidation of guanine and guanosine, is found in rabbit liver [Schmidt (87)]; it can be separated from an accompanying enzyme which liberates

ammonia from guanylic acid. The desamidation of guanine is not, therefore, as in the case of adenine, dependent on its linkage with carbohydrate.

Another specific desamidase, asparaginase, is found in yeast [Grassmann & Mayr (88)]. Its action consists solely in splitting ammonia from asparagine and aspartic acid diamide. The enzyme differs from dipeptidase.

Virtanen & Tarnanen (89) have isolated an enzyme from bacteria which regulates the equilibrium between synthesis and decomposition in the system, aspartic acid $\rightleftharpoons$ fumaric acid and ammonia. This enzyme, called aspartase, is strictly specific. Synthetic action, for instance, is prevented even by the addition of a methyl group to fumaric acid; the pH optimum of the enzyme is approximately at neutrality. Aspartase is found in bacteria and in higher plants, but not in yeast nor in animal organs.

Carbohydrases

a) *Polyases.*—Waldschmidt-Leitz & Reichel (90) have furthered the discussion of the chemical nature of pancreatic amylase by pointing out that it is possible by very simple methods to liberate the enzyme, in the process of purification, from every trace of protein. Only a very small fraction of the amylase in the pancreas occurs in insoluble form as desmo-enzyme [Willstätter & Rohdewald (91)]. The enzyme becomes gradually inactive in water-free glycerine, but is not inhibited by glycerine which contains water. Remarkable results have been achieved [Willstätter & Rohdewald (92)] in experiments on the components of the amylytic system of the white blood corpuscles. Here eight enzymes, differing from each other and all belonging to the α-amylase type, have been obtained and differentiated according to their solubility in glycerine, their inhibition by glycerine, and their independence of phosphate. Their differences are explained by changes in the molecular or colloidal state of the bearer of the enzymatically active group.

The formation and properties of α- and β-amylases in germinating grain, the differentiation and separation of which were obtained by adsorption agents [Waldschmidt-Leitz, Reichel & Purr (93)], have been frequently examined. According to Nordh & Ohlsson (94) dormant barley contains only β-amylase [Doby & Burger (94*a*)] (potato amylase); Doby & Brázay (94*b*) (sugar beet amylase)]. It increases regularly in quantity when germination sets in, whereas

α-amylase appears only during germination. Similar conditions hold for rye and oats [Ohlsson & Uddenberg (95); Ohlsson & Edfeldt (96)]. The increase of amylolytic activity during germination is not, however, a fresh enzyme formation, but is due to an activation (by amylokinase) [Myrbäck & Myrbäck (97)]. Besides this, an increase in the soluble part of the amylase is observed, especially in the case of the β-component, which increase is based on proteolytic decomposition. In leaves, too, amylase seems to be present, for the most part in an inactive and insoluble form [Oparin & Risskina (98)]. Methods for the selective destruction of α- and β-amylase from malt have been described by van Klinkenberg (99); α-amylase is inhibited by α-maltose, β-amylase only by β-maltose. Starch itself is said to consist of a mixture of one part of α- and two parts of β-starch, only the α-component, which can be decomposed by α-amylase, being responsible for the iodine coloring [van Klinkenberg (100)]. α-Amylase is specifically adsorbed by crude starch and in this manner it can be separated from β-amylase [Holmbergh (101)]. Chrzaszcz & Janicki (102) have found a natural inactivator of amylase in germinating buckwheat and have called it "sisto-amylase." It increases in quantity during germination. The substance appears to be protein in character. Its inhibitory action on amylase seems to be due to adsorption of the enzyme. Amylase activity at germination is therefore regulated by the quantity of "sisto-amylase" and of "eleuto-amylase," a substance similar to peptone and antagonistic to "sisto-amylase"; so also is the proportion of "saccharifying" and "liquefying" power. The inactivation of plant amylase, when heated in solution, seems to be mainly due to the coagulation of accompanying proteins [Oparin & Manskaja (103); Oparin, Manskaja & Magaram (104)]; it can be prevented by peptone. Pringsheim, Borchardt & Hupfer (105) report on the specific activation of α-amylase by oxidised glutathione which occurs in the more advanced stages of starch degradation.

Extensive studies leading to new results have been carried out on the specificity of the cellulase and similar enzymes which split polysaccharides [Grassmann, Zechmeister, Tóth & Stadler (106); Grassmann, Stadler & Bender (107)]. According to these, mannanase, xylanase, inulinase, and cellulase are to be regarded as absolutely specific enzymes. In molds, along with cellobiase and cellulase, a xylanase has been found which can be separated by adsorption agents. It has also been possible to remove cellobiase (β-gluco-oligo-

saccharase) by adsorption with aluminum-mono-hydrate from the mixture with cellulase (β-gluco-polysaccharase). The specificity limits of both enzymes have not been very sharply defined, but they lie about at cello-hexa-ose. Cellulase does not split the cellulose chains from the ends, and no glucose is formed. The enzyme is therefore to be distinguished from the β-glucosidase of emulsin. Other reports have also been made of the separation of cellulase and cellobiase from bacteria extracts by adsorption [Trager (108)].

A recently detected enzyme which plays a part in the decomposition of pectin is described as pectolase. It is found in emulsin and mold preparations but not in yeast [Ehrlich (109)]. Particularly active preparations can be obtained from *Penicillium* species and from the digestive tract of the vineyard snail. The action of this enzyme consists in splitting the ring-shaped pectolic acid (tetragalacturonic acid C) by way of the open-chain pecto-lactonic acid to the monomolecular galacturonic acid. A weak acid reaction is described as the activity optimum.

b) *Hexosidases.*—Helferich and co-workers (110) have undertaken numerous researches on the purification and specificity of the β-glucosidase of emulsin. The enzyme has been purified by fractional precipitation with silver oxide up to the glucosidase value of 10. Summarising the present state of specificity experiments, we find that the β-glucosidase is apparently also responsible for splitting β-galactosides. The theory, therefore, of the existence of specific glucosidases in Weidenhagen's sense (111) appears to be untenable. At the same time, emulsin is found to contain an α-glucosidase, though in smaller quantity, to which is ascribed the observed splitting of α-glucosides and probably also of α-galactosides. The β-glucosidase of emulsin further splits α-mannosides and derivatives of iso-rhamnose. We must bear in mind the possibility that the primary condition for the cleavability of a glucoside by β-*d*-glucosidase is the trans-position of the oxygen atoms at carbon atoms 1 and 2; accordingly the enzyme should be called trans-aldo-pyranosidase. For preparations of β-glucosidase from lactose yeasts, the relation between the cleavability of β-glucosides and β-galactosides differs from that in emulsin [Hofmann (112)]. Attempts to define the chemical nature of the enzyme in emulsin have led to no clear result [Tauber (113)]; no enzyme preparations were obtained free from protein.

The specificity of α-glucosidase has also been studied. The state-

ments have been made that autolysates from *Aspergillus* cultures [Myrbäck (114)], as well as extracts from breast glands [Kleiner & Tauber (115)] can break down maltose but not α-methyl-glucoside. Weidenhagen (116) points out, in defense of his theory of the specificity of the hexosidases, that such findings are delusive results arising from the relatively difficult cleavability of α-methyl-glucoside; he asserts that the theory of the identity of maltase and α-glucosidase still holds [for the contrary, see Pringsheim & Loew (117)]. Willstätter & Rohdewald (118) have found in leucocytes, along with a form soluble in glycerine, an insoluble desmo-maltase which can be obtained in glycerine solution by decomposition of the cell residues by proteolysis.

The liberation of saccharase from yeast can be achieved according to Grassmann & Peters (119) by a proteinase of the papain type and also by plant amylase. The process in question is hydrolysis of the cell membrane. Saccharase is, therefore, an endo-enzyme, its insolubility being due to mechanical anchoring in the cell structure, and not to chemical linkage to protoplasmic substance as in the case of the desmo-enzymes [Willstätter & Rohdewald (120)]. According to Weidenhagen (121), saccharase, as β-*h*-fructosidase, is also responsible for the splitting of inulin, though this takes place much more slowly than that of cane sugar.

Oxidative Enzymes

a) *Catalases.*—Stern (122) has conducted a series of experiments on the properties of animal catalase. His results support the view that catalase of the liver is identical with that of the red blood corpuscles; from the diffusion capacity a molecular weight of 69,000, similar to that of haemoglobin, was deduced. But the bearer of the catalase, protein in nature, to which a special fluorescence is ascribed, seems to be different from the protein of haemoglobin, the isoelectric point being different. An examination of the mechanism of the catalase reaction leads to the theory of a primary degradation of hydrogen peroxide into two hydroxyl radicals [Haber & Willstätter (123)].

b) *Peroxidases.* — Important new experiments on peroxidases deal with the chemical nature of the enzyme of horse radish, the active group of which had been described as an iron-porphyrin compound on the basis of the absorption spectra of purified preparations [Kuhn, Hand & Florkin (124)]. In opposition to this, Elliott &

Keilin (125) have proved that, in purer peroxidase preparations, there is no simple relation between the haematin content and the activity of the peroxidase, and that the entire quantity of haematin present in the peroxidase is free haematin. Neither is peroxidase inhibited by carbon monoxide [Elliott & Sutter (126)]. The question of the chemical nature of the peroxidase is, therefore, still unsolved, and also the statements on the nature of the catalase [Zeile & Hellström (127)] will require further testing.

c) *Uricase.*—As a probable intermediary product in the enzymatic formation of allantoin from uric acid, Schuler & Reindel (128) postulate hydroxy-acetylene-diurein-carbonic acid, according to the oxidation process by MnO_2 [Kleinmann & Bork (129)]. Liver and kidney participate in the synthesis of uric acid by birds; it proceeds by way of a still-unknown intermediate, which is not of purine character. Amino acids serve as the source of nitrogen for the formation of uric acid [Schuler & Reindel (130)].

Fermentation Enzymes

Objections have been raised to the proposed uniformity in nomenclature of the activators participating in fermentation processes [Neuberg & Euler (131)] on the grounds that such uniformity is impossible so long as confusion still exists concerning the functions of the different activators [Kluyver & Stheeman (132)]. The chemical nature of co-zymase has not yet been completely defined. The fact that it is broken down by phosphatase [Myrbäck (133)] confirms the assumption of its nucleotide character. But it does not appear to be identical with any of the familiar nucleotides, nor with adenosine-triphosphoric acid isolated from muscle [Euler & Nilsson (134)], which has the constitution of an amino-pyrophosphoric acid (of adenine) [Barrenscheen & Filz (135)].

Like the existence of cell-free fermentation [Belitzer & Gorkin (136)], the separability of the lactic-acid-forming enzyme system in erythrocytes from the living cell has been experimentally proved [Meyerhof (137)]. Here, as in alcoholic fermentation, the degradation of carbohydrates proceeds by way of labile phosphoric-acid esters. Contrary to numerous cases recorded in the literature, in which the intermediary formation of sugar-phosphoric esters took place, the transformation of sugar in fermentation, according to Nord (138), occurs without previous phosphorylation; the presence of phosphates is not necessary.

Zuckerkandl & Messiner-Klebermass (139) have examined the part played by iron in alcoholic fermentation. They differentiate two iron-containing enzymes in yeast which are mutually complementary: a system which catalyses respiration and is sensitive to cyanide, and a system relatively resistant to cyanide which is active during fermentation. In contrast to alcoholic fermentation, butyric fermentation is inhibited by hydrocyanic acid and also by carbon monoxide. One of the enzymes participating in butyric fermentation, therefore, seems to be a heavy metal compound [Kempner (140)].

Quastel & Wheatley (141) have drawn attention to relations between sulfhydryl compounds and the fermentation system. They assert that the ratio of fermentation to respiration in baker's yeast is altered by cysteine or glutathione so that fermentation is accelerated while respiration is reduced. The inhibition of alcoholic fermentation by iodoacetic acid is also said to be due partly to the elimination of sulfhydryl by that acid [Schroeder, Woodward & Platt (142)]. Auhagen & Neuberg (143) on the other hand, using yeast, describe a replacement, under the influence of glutathione, of alcoholic fermentation by lactic fermentation. This is also observed when plasmolytic agents are present or when the quantity of the enzyme is greatly reduced. Lohmann (144) was the first to prove that a sulfhydryl compound, reduced glutathione, functions as a specific activator of the formation of lactic acid from methylglyoxal by way of glyoxalase. Glutathione does not act here as the member of a complex with heavy metal. Glycogen seems on the other hand to be split up into lactic acid even in the absence of glutathione. The degradation of pyruvic acid to lactic acid by muscle extract is also considerably accelerated by sulfhydryl compounds [Barrenscheen & Beneschovsky (145)]. The inhibitory action of iodoacetic acid on the formation of lactic acid, which has also been observed in the case of bacteria [Meyer (146)], is, according to Lohmann (147) only partially, and according to Dickens (148) completely, neutralised by reduced glutathione. This holds also for the action of anti-glyoxalase, isolated from pancreas, which is held to attack the glyoxalase system by reacting with reduced glutathione (149).

A component enzyme of the alcoholic fermentation system, carboxylase, has been fractionated with methyl alcohol and tannin by Langenbeck and co-workers (150) and purified approximately 1,000 times. In the process, saccharase, maltase, and proteinase were separated. Carboxylase, free from zymase, has been shown to be

present in *B. Bordeaux*, an acid-former [Neuberg & Simon (151)]. Carboxylase is also found in higher plants, e.g., in plant seeds (peas) [Andersson (152)], or in green leaves [Wetzel (153)]; but the enzyme seems to be present here mostly in inactive form. It is activated by co-carboxylase from yeast [Auhagen (154)].

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THE CHEMISTRY OF THE ACYCLIC CONSTITUENTS OF NATURAL FATS AND OILS*

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The chemistry of the constituents composing naturally occurring phosphatides, waxes, glycerides, fatty acids, alcohols, and hydrocarbons has received much attention from investigators during the past two years. Chemical, biochemical, physiological, and physical methods have been applied in the experimental studies, and many new compounds have been synthesized. An immense amount of new data has accumulated, and the reviewer confesses that it is impossible, in the space that is available, to touch upon more than a small fraction of the works that have been published.

Component Fatty Acids of Phosphatides

The unsaturated fatty acids of brain phosphatide have been investigated by Klenk (1). The separation of individual fatty acids was accomplished by means of fractional distillation of the methyl esters *in vacuo* using a high column. The acids of the C_{18} series consisted almost entirely of oleic acid while two members of the C_{20} series were found, namely, $C_{20}H_{38}O_2$ and $C_{20}H_{32}O_2$. The acids of the C_{22} series were highly unsaturated and, on bromination, an ether-insoluble bromide corresponding to the formula $C_{22}H_{34}Br_{10}O_2$ was isolated.

The highly unsaturated acids of beef brain have also been investigated by Brown (2) and were found to belong mainly to the C_{22} series. The acid $C_{22}H_{34}O_2$ predominated, but a small amount of the acid $C_{22}H_{36}O_2$ was also found. Arachidonic acid could not be identified, but a small amount of an unsaturated tetracosanic acid, probably $C_{24}H_{38}O_2$, was also present.

The phosphatides of human brain have been studied by Yokoyama & Suzuki (3). The mixed phosphatides were separated by means of the cadmium chloride double salt into α- and β-lecithin. The α-lecithin on hydrolysis was found to contain oleic, arachidonic, and palmitic acids, while β-lecithin yielded oleic, linoleic, arachidonic,

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and palmitic acids. Nishimoto & Suzuki (4) have described the separation, purification, and analyses of α- and β-cephalins of the human brain.

A comparative study of the fatty acids of the phosphatides and of the neutral fat of beef liver has been reported by Klenk & Schoenebeck (5). It was found that the unsaturated fatty acids of the phosphatides corresponded approximately to those of the brain phosphatides, although certain differences were noted. The unsaturated acids of the C_{18} series contained an appreciable amount of linoleic acid, while the C_{20} acids were more highly unsaturated than those of the C_{22} series. In the neutral fat of the liver unsaturated acids of the C_{20} and C_{22} series were present but in smaller amounts than in the phosphatides.

Klenk (6) has also reported analyses of the phosphatides and oils obtained from the liver of *Etmopterus spinax*, a small species of shark. The liver oil contained more than 50 per cent of unsaponifiable matter which consisted largely of squalene. The phosphatides contained a small amount of saturated acids and a large amount of highly unsaturated acids belonging to the C_{18} and C_{22} series. The unsaturated fatty acids obtained from the neutral fat on saponification were separated by fractionation of the methyl esters. The fractions were hydrogenated and, after saponification, acids of the C_{16}, C_{18}, C_{20}, C_{22}, and C_{24} series were obtained. Comparative analyses have been made by Klenk (7) of the fatty acids of the phosphatides and oil from the liver and the body fat of the frog. The phosphatide fatty acids after fractionation of the methyl esters and hydrogenation were found to consist of acids of the C_{16}, C_{18}, C_{20}, and C_{22} series.

The cephalin fraction from human brain has been investigated by Bülow & Page (8) and by Page & Rudy (9). The results obtained corroborate mainly the findings reported by Klenk. Attention has been called by Thannhauser & Fränkel (10) to the occurrence of notable amounts of nitrogen in the unsaponifiable matter obtained from the liver fat, and these authors have shown that the nitrogen is present in the form of lignoceryl sphingosine. Fränkel & Bielschowsky (11) propose the name "ceramide" for this class of compounds and they showed that lignoceryl sphingosine could be isolated directly by extraction with solvents from pig's liver. According to Fränkel, Bielschowsky & Thannhauser (12), pig's liver also contains a polydiaminophosphatide which is believed to consist of combined palmityl, stearyl, and lignoceryl sphingomyelins. In

this connection it should be mentioned that Klenk (13) has recently published the results of an investigation concerning the composition of the sphingomyelin obtained from beef heart muscle. The cleavage products consisted of lignoceric and stearic acids and sphingosine.

A study of the metabolism of the phosphatides in the rat on fat-poor and fat-rich diets as measured by the change in the iodine number of the component fatty acids has been reported by Sinclair (14). In an extensive study of the composition of the component fatty acids of the phosphatides of beef liver by Snider & Bloor (15) it was found that the ratio of unsaturated to saturated acids was about 55 : 40. The unsaturated acids contained representatives of the C_{18} and C_{20} series, the highly unsaturated members predominating. The amounts found were as follows: 21 per cent of oleic, 45 per cent of linoleic, and 31 per cent of arachidonic acid. The saturated acids were composed of 17 per cent of stearic and 29 per cent of palmitic acid.

An interesting study of the sulfur-containing lipids of the brain has been reported by Blix (16). The sulfatide is apparently a sulfuric-acid ester of cerebron, since the cleavage products obtained on hydrolysis consist of sulfuric acid, cerebronic acid, sphingosine, and galactose.

According to King & Dolan (17), lysolecithin is hydrolyzed more rapidly than lecithin by enzyme preparations from the intestinal mucosa and from bone.

Smith & Chibnall (18) have studied the composition of the phosphatides contained in cocksfoot grass. A mixture of lecithin and cephalin was isolated, together with salts of phosphatidic acid. The phosphatides contained both linoleic acid and linolenic acid, together with saturated fatty acids. Jordan & Chibnall (19) have investigated the distribution of phosphatides and phosphatidic acid in the runner bean (*Phaseolus multiflorus*).

Some new information regarding the composition of bacterial phosphatides has been published during the past two years. Anderson & Uyei (20) examined the phosphatide isolated from the leprosy bacillus. The substance was a stable amorphous powder, which melted with decomposition at 231° and contained 1.75 per cent phosphorus but only a trace of nitrogen. The cleavage products obtained on hydrolysis were similar to those of the phosphatides from other acid-fast bacteria. The fatty acids amounted to 58 per cent and consisted of palmitic acid, two unsaturated acids of the C_{16} and

C_{18} series, a liquid saturated fatty acid similar to tuberculostearic acid, and two other unidentified acids of high molecular weight. The water-soluble constituents were glycerophosphoric acid, inosite, mannose, and another hexose sugar. Chargaff (21) isolated from the diphtheria bacillus a small quantity of a phosphatide which contained 1.4 per cent of phosphorus and 0.8 per cent of nitrogen. On acid hydrolysis the substance yielded reducing sugars and a mixture of fatty acids all of which could not be completely identified, but palmitic acid and a higher hydroxy acid named "corynin," $C_{50}H_{100}O_4$ (m.p. 70–71°), were isolated. The same author (22) has also investigated the composition of the phosphatide, wax, and acetone-soluble fat contained in the Calmette-Guérin bacillus (BCG). This organism was found to be richer in lipoids than strains of the bovine and human tubercle bacillus cultivated under identical conditions. Chargaff & Dieryck (23) have published a very comprehensive comparative study of the lipoid content of 25 different strains of tubercle bacilli.

Newman & Anderson (24) have investigated the composition of the phosphatides of yeast and determined the cleavage products which are obtained on hydrolysis. The phosphatide contained 4.22 per cent of phosphorus and 1.78 per cent of nitrogen; the ratio of N : P = 1 : 1.07. As judged by the proportion of total nitrogen to amino nitrogen the phosphatide was a mixture containing approximately four parts of lecithin and one part of cephalin. The saturated fatty acids consisted of about equal parts of palmitic and stearic acids. The unsaturated acids contained acids of the C_{16} and C_{18} series and on catalytic reduction gave about 60 per cent of palmitic and 40 per cent of stearic acid, together with a trace of lauric acid. The water-soluble constituents were glycerophosphoric acid, choline, and aminoethyl alcohol. The phosphatide did not contain any carbohydrate.

Constituents of Waxes

Chibnall and collaborators have carried out elaborate investigations of the constituents of the waxy coating of apple cuticle (25) and of brussels sprouts (26), having first worked out an excellent method for separating paraffins, ketones, higher secondary, and higher primary alcohols, which are frequently found occurring in such vegetable waxes. The wax of the apple peel contained *n*-hepta-

cosane, *n*-nonacosane, *d*-10-nonacosanol, *n*-hexacosanol, *n*-octacosanol, and triacosanol. The wax from brussels sprouts contained *n*-nonacosane, 15-nonacosanone, 15-nonacosanol, ceryl alcohol, cerotic acid, and probably *n*-hentriacontane. In this connection mention should be made of the study reported by Piper, Chibnall & others (27) on the synthesis and crystal spacings of paraffins from $C_{25}H_{54}$ to $C_{36}H_{74}$, of long-chain ketones, and of secondary alcohols. The wax from apple peels has also been examined by Sando and collaborators (28), who found principally nonacosane and 10-nonacosanol.

Karrer & Schwarz (29) obtained an ether-soluble wax, m.p. 76–78°, from the resinous coating of *Sarcocaulon rigidum* Schinz., which on saponification yielded ceryl alcohol and ferulic acid. Jermstad (30) isolated a higher alcohol called sorbol (m.p. 78°), probable formula $C_{34}H_{70}O$, from a wax-like substance obtained from the berries of *Sorbus aucuparia*. A wax (m.p. 78–80°) was obtained by Sakurai (31), which on saponification gave lauric, palmitic, stearic, and hydroxypalmitic acid but no alcohol.

The wax obtained from *Psylla* and *Schizoneura* has been examined by Schulz & Becker (32). Kono (33) has reported upon the composition of the wax of *Ceroplastes ceriferus* And. and *Ceroplastes floridensis* Comst., while Ikuta (34) has studied the fatty acids contained in Japanese beeswax. The dibasic acids in Japan wax (35) and in the wax from sumac berries (36) have been investigated by Tsujimoto.

Glycerides and Their Constituent Fatty Acids

Comprehensive analyses of the component fatty acids and the glyceride structure of a number of fats and oils have been published by Hilditch and collaborators. The composition of the fatty acids and glycerides of the following seed fats and oils was determined: *Allanblackia Stuhlmannii, Pentadema butyracea, Butyrospermum parkii* (Shea), and *Vateria indica* (Dhupa) (37); *Myristica malabarica* (38); commercial palm oils (39); the body fats of the pig (40); milk fats of Indian goats and sheep (41); certain oleo oils (42); fruit coat fats (43); partially hydrogenated fats (44); and of butter fats (45). Examinations were also made of certain azelaoglycerides obtained by oxidation with permanganate in acetone solution of synthetic and natural glycerides (46). In a study of the composition

of ointments discovered in ancient Egyptian tombs (47), one of which was perhaps 5,000 years old, it was found that glycerides were still present. The oleic acid content was low, probably due to gradual atmospheric oxidation, but the normal oxidation products of this acid, azelaic and nonoic acids, were present.

The presence of linoleic and linolenic acids, 0.2 and 0.1 per cent, in the fatty acids obtained from butter fat was indicated by a study of the bromides reported by Eckstein (48). Bosworth & Brown (49) in fractionating large quantities of fatty-acid esters prepared from butter fat were unable to identify linoleic acid with certainty, although highly unsaturated acids of the C_{20}, C_{22}, and C_{24} series were found. These authors also report the presence of unsaturated decenoic acid, $C_{10}H_{18}O_2$, and tetradecenoic acid, $C_{14}H_{26}O_2$, in butter fat.

The analyses of the glyceride fatty acids of cocksfoot grass and perennial ryegrass reported by Smith & Chibnall (50) show that the mixed acids are highly unsaturated, containing linoleic and linolenic acids, the saturated acids being mainly palmitic and stearic acids. The oil obtained from commercial lycopodium has been investigated by Riebsomer & Johnson (51). The fatty acids consisted mainly of oleic acid, 55–60 per cent, and 9-10-palmitoleic acid, 30–35 per cent; hence, the acid described by Langer under the name of lycopodiumoleic acid, is apparently a mixture of these two acids. Other acids identified were palmitic acid, linoleic acid, and 9-10-dihydroxystearic acid (m.p. 93–93.5°).

The constants and composition of wheat-germ oil have been determined by Jamieson & Baughman (52), of oiticia oil by van Loon (53), and of hazelnut oil by Schuette & Chang (54). The seed fat of the California bay tree (*Umbellulatia Californica*), containing mainly glycerides of capric and lauric acids, is an excellent source for capric acid according to Noller, Millner & Gordon (55).

The compositions of a large number of fish oils have been reported. Cox & Reid (56) examined the oil of *Ruvettus pretiosus*, the "castor oil fish," and found that it corresponded to a liquid wax containing about equal parts of fatty acids, mainly oleic acid, and unsaturated and saturated alcohols such as oleyl, cetyl, and octadecyl alcohols. Tsujimoto (57) has reported upon the examination of a large number of liver oils of elasmobranch fish. These oils contained hydrocarbons such as pristane, $C_{19}H_{38}$, and squalene, $C_{30}H_{50}$, together with chimyl, batyl, and selachyl alcohols and selacholeic acid, $C_{24}H_{46}O_2$. The same author has also investigated the fatty

acids and a hydrocarbon found in the liver oil of ishinagi (*Stereolepis ischinagi*) (58). The oil obtained from the eggs of the shark, as reported by Ono (59), contained 21 per cent of saturated and 78 per cent of unsaturated fatty acids, while the unsaponifiable matter contained cholesterol, cetyl, octadecyl, and selachyl alcohols. An analysis of the oil obtained from *Clupea ilisha*, a fish found in the rivers of Bengal and Burma, has been reported by Goswami and Datta (60). Rees (61), in an analysis of bream oil, isolated a small quantity of lauric acid but could not find any lauric acid in dog-fish oil.

Several unusual vegetable fats and fatty-acid esters have been analyzed. Steger & van Loon (62) examined the seed fat of *Picramnia sow* and found 4.6 per cent of saturated acids and about 90 per cent of 6-7-stearolic acid (tariric acid). Taylor & Sherman (63) found that the fatty acids associated with corn starch occur only in the α-amylose, while β-amylose appears to be pure carbohydrate, free from fatty acids. The occurrence of a monostearic acid ester of a triterpenediol (m.p. 124°) in the fruit of *Erythroxylon novogranatense* has been reported by Zimmermann (64). The free diol (m.p. 231°) has apparently the formula $C_{30}H_{50}O_2$ or $C_{30}H_{52}O_2$. A new unsaturated acid (m.p. 85–86°), an isomer of eleostearic acid, has been isolated from the oil of "akarittom," *Parinarium laurinum* (65). The fat extracted from digitalis leaves, as reported by Schwarz (66), gave on saponification myristic, palmitic, cerotic, melissic, oleic, linoleic, and linolenic acids, while the unsaponifiable matter contained melissyl alcohol, sitosterol, and triacontane. Ergosteryl palmitate (m.p. 106–108°; $[\alpha]^{20}_{5461} - 67°$), has been identified by Oxford & Raistrick (67) as a metabolic product produced by 15 strains of *Penicillium brevi-compactum*. The ether-soluble constituents of *Cascara sagrada* contain, according to Jermstad (68), palmitic, arachidic, melissic, oleic, linoleic, and linolenic acids, together with rhamnol, $C_{20}H_{36}O$ (m.p. 132–33°), and phytosterol. The unsaponifiable matter of shea fat contains, according to Hopkins & Young (69), an unsaturated hydrocarbon, m.p. 64°, corresponding to the formula $C_{32}H_{56}$. Apparently the same substance has been described under the name of karitene by Bauer & Umbach (70), who found the melting-point to be 63°, but who regard the hydrocarbon to be an isoprene derivative. Lüning & Drude (71), after examining several samples of cocoa butter, report that they were unable to detect arachidic acid or any acid higher than stearic acid in this material. The presence

of arachidic acid in supposed cocoa butter is regarded as indicating adulteration with hardened peanut oil.

A number of synthetic glycerides have been prepared and their properties and constants determined by King and collaborators (72), and also by Verkade, van der Lee & Meerburg (73). Higher fatty-acid esters of cellulose (74) have also been prepared.

The fat obtained from yeast has been examined by Weiss (75), who reports that various higher oxygenated acids are present as well as an optically active valeric acid. The acetone-soluble fraction of the fat extracted by alcohol and ether from fresh living yeast cells has been examined by Newman & Anderson (76). The saturated acids amounting to 18 per cent consisted of a mixture of approximately 75 per cent of palmitic acid and 25 per cent of stearic acid, together with a trace of an acid lower than palmitic acid. The liquid unsaturated acids amounting to 78 per cent had an iodine number of 119 and on catalytic reduction gave a mixture of acids containing 25 per cent of palmitic acid and 75 per cent of stearic acid. No acid higher than stearic acid could be found. The unsaponifiable matter amounted to 46.7 per cent of the fat and contained a small amount of yeast sterols, but the bulk of the material consisted of a mixture of saturated hydrocarbons, evidently introduced accidentally during the cultivation of the yeast. The water-soluble fraction obtained on saponification of the fat amounted to 5.9 per cent and consisted of glycerol.

Uyei & Anderson (77) extracted more than 4 kg. of the leprosy bacillus with alcohol-ether and chloroform and separated the lipoid material, which amounted to 18.7 per cent, into phosphatide, acetone-soluble fat, and wax. The acetone-soluble fat isolated from the timothy grass bacillus has been analyzed by Pangborn, Chargaff & Anderson (78). The fat was evidently not a glyceride since glycerol could not be identified. The fatty acids represented a mixture of saturated solid acids, principally palmitic acid, together with a trace of a higher optically active hydroxy acid, unsaturated acids which on catalytic reduction gave palmitic acid, and a liquid saturated acid very similar in composition and properties to tuberculostearic acid. The unsaponifiable matter, which amounted to 27 per cent of the fat, was an unsaturated oil with an iodine number of 126 which did not give any sterol color reactions.

Chargaff (79) has analyzed the acetone-soluble fat of the bacillus Calmette-Guérin (BCG) and found that the solid fatty acids con-

sisted largely of palmitic acid, together with a small amount of cerotic acid. The unsaturated acid was probably oleic acid since on catalytic reduction stearic acid was obtained. About 9 per cent of the fat consisted of liquid saturated acids of high molecular weight similar to those found in other acid-fast bacteria. The unsaponifiable matter gave no sterol color reactions.

It is evident from the work reported during recent years that the acetone-soluble fats from acid-fast bacteria consist of neutral fat, together with nearly 30 per cent of free fatty acids. In the older literature such fats were referred to as glycerides, although glycerol had never been definitely identified. In the more recent analyses of such bacterial fats it was found that from 5 to 6 per cent of water-soluble constituents were liberated on saponification but no glycerol could be isolated from this material, some substance other than glycerol being present. For this reason it was assumed that the neutral fats of acid-fast bacteria were esters of fatty acids with higher alcohols or possibly with carbohydrates. It was shown during the past year by Anderson & Newman (80) that the acetone-soluble fat from the human tubercle bacillus contained the crystalline disaccharide trehalose in place of glycerol. It is apparent therefore that the acid-fast bacteria, although the chief constituent of the medium in which they are grown is glycerol, build up their fats on a decidedly different pattern from the usual plant and animal cells.

Individual Fatty Acids

The constitution of cerebronic acid has been reinvestigated by Levene and collaborators and by Klenk & Diebold. The conclusions of these investigators are not in agreement, hence the question as to the constitution of cerebronic acid as well as the exact nature of the fatty acids obtained by oxidation appears to be in about the same state as before. According to Taylor & Levene (81), Levene & Heymann (82), and Levene & Yang (83), cerebronic acid, $C_{25}H_{50}O_3$, is probably not a homogeneous substance but represents a mixture of hydroxy pentacosanic acids which on oxidation with permanganate in acetone solution yields several higher fatty acids, including some 32 per cent of a tetracosanic acid, $C_{24}H_{48}O_2$ (m.p. 80.5–82°). On the other hand Klenk & Diebold (84) regard cerebronic acid as α-hydroxytetracosanic acid, $C_{24}H_{48}O_3$, which on oxidation according to

Levene's method yields tricosanic acid, $C_{23}H_{46}O_2$, together with certain lower homologues formed by further oxidation of the tricosanic acid.

In order to secure some evidence for or against Klenk's assumption that lower homologues were formed during the oxidation from tricosanic acid, Levene & Yang (85) subjected α-hydroxystearic acid to oxidation with permanganate in acetone solution. The only oxidation product found was margaric acid, $C_{17}H_{34}O_2$.

Chargaff (86) has synthesized the following higher fatty acids of the C_{18} and C_{26} series with branching chains, these acids being isomers of tuberculostearic acid and phthioic acid: α-ethyl-*n*-hexadecanoic acid (m.p. 37.5–38°); α-ethyl-*n*-tetracosanic acid (m.p. 65.5°); α-*n*-butyl-*n*-docosanic acid (m.p. 60–61°); α-*n*-hexyl-*n*-eicosanic acid (m.p. 65°); α-*n*-decyl-*n*-hexadecanoic acid (m.p. 54°), and α-*n*-dodecyl-*n*-tetradecanoic acid (m.p. 70–71°). The melting-points of the synthetic acids were much higher than those of the isomeric natural acids.

Fordyce & Johnson (87) have synthesized isostearic acid, 16-methyl-heptadecanoic acid (m.p. 67.6–68.2°); isopalmitic acid, 14-methyl-pentadecanoic acid (m.p. 61.8–62.4°), and isomyristic acid, 12-methyl-tridecanoic acid (m.p. 50.5–51°). The synthesis of ι-*m*-cresoxystearic acid has been reported by Niederl & Liotta (88). This acid yields by intramolecular rearrangement the isomeric ι-2-hydroxy-4-methyl phenyl-stearic acid, which shows fatty acid as well as phenolic characteristics and possesses interesting physiological properties. Bleyberg & Ulrich (89) have synthesized the normal C_{20}, C_{22}, C_{24}, C_{26}, C_{28}, and C_{30} acids and determined their constants.

In the examination of the fat, obtained by extraction with solvents from *Lactobacillus acidophilus*, it was found by Crowder & Anderson (90) that an appreciable amount of an optically active dihydroxystearic acid could be isolated. The acid was present in the free state and after purification melted at 106–107°. The highest observed rotation was $+7.78°$ but the acid was easily racemized. On reduction with hydriodic acid, stearic acid was obtained.

In the purification of phthioic acid isolated from the acetone-soluble fat of the human tubercle bacillus by repeated fractionation of the methyl ester in a high vacuum, Anderson (91) obtained a new levorotatory acid. The purified phthioic acid, a thick oil at ordinary temperature, corresponded in composition to the formula $C_{26}H_{52}O_2$; $[\alpha]_D = +11.41°$. The composition of the levorotatory acid agreed

approximately with the formula $C_{30}H_{60}O_2$. The acid melted at 48–50°; $[\alpha]_D = -6.14°$.

Rudy (92) has described alpha and beta forms of the octabromide of behenic acid obtained on bromination of the unsaturated fatty acids from brain cephalin. A series of new esters of hexabromostearic acid have been prepared by Stanfield & Schierz (93).

Addition compounds of maleic anhydride with α- and β-eleostearic acid and their oxidation products have been studied by Morrell & Samuels (94). The rates of oxidation of monolayers of α- and β-eleostearic acid and other unsaturated acids have been determined by Hughes & Rideal (95). Studies of the isomerism, polymerization at ordinary temperature, and polymerization at elevated temperatures of eleostearic acid have been published by Rossmann (96).

The resolution of dihydroxystearic acids by means of the strychnine salts into the *d*- and *l*- forms was reported by Inoue & Suzuki (97). The optical activity of 12-hydroxystearic acid, prepared by hydrogenation of ricinoleic acid, and of a series of esters prepared from 12-hydroxystearic acid has been determined by Straus, Heinze & Salzmann (98), while the optical activity of purified ricinoleic acid has been determined by André & Vernier (99).

A detailed study of the products formed in the "elaidin" reaction with oleic, petroselinic, and erucic acids has been reported by Griffiths & Hilditch (100) and the same reaction with erucic acid has been studied by Rankoff (101). A series of esters were prepared from oleic and elaidic acids and their constants were determined by Koyama (102). The constitution of oleodibromostearic acid and elaidodibromostearic acid has been investigated by Maruyama & Suzuki (103).

Products resulting from the oxidation of highly purified oleic acid by gaseous oxygen under various conditions have been determined by Skelton (104). Smedley-Maclean & Pearce (105) have studied the course of oxidation of oleic acid by hydrogen peroxide with and without the addition of copper sulfate.

The structure of couepic acid (106) has been discussed by Rossmann (107), who regards the acid as containing a cyclobutane ring but van Loon & Steger (108) believe that the properties and reactions of couepic acid are more in accord with those of an isomer of eleostearic acid.

Grossfeld (109) has discussed the limitations of the lead-salt method of separating the higher saturated fatty acids from the liquid

unsaturated acids in comparison with the permanganate oxidation method. The use of monoureides as suitable derivatives for the characterization of fatty acids has been suggested by Stendal (110). For the preparation of the ureides the ethyl esters of the acids are treated in pyridine solution with urea and alcoholic sodium ethoxide. The ureides are said to be well suited for the determinations of molecular weights and they are easily hydrolyzed for the recovery of the free acid.

Hinegardner (111) has described the racemization of chaulmoogric and hydnocarpic acids. It was found that the amides of the active acids on distillation *in vacuo* with phosphorus pentoxide yielded the inactive nitriles which on hydrolysis gave the inactive acids. The melting-points of the inactive acids were identical with those of the dextrorotatory acids.

High-Pressure Hydrogenation

Several additional publications have appeared on the subject of high-pressure hydrogenation. The formation of alcohols, glycols, or hydrocarbons under different conditions and with different catalysts has been investigated by Folkers & Adkins (112) and by Adkins, Wojcik & Covert (113). The production of higher alcohols, ketones, etc., by high-pressure hydrogenation of fatty acids, aldehydes, esters, and glycerides has been discussed by Normann (114), Oda (115), and by Tanaka and collaborators (116).

Higher Alcohols

Several higher aliphatic alcohols have been referred to previously in this article in the paragraph on waxes. It was mentioned in the first volume of the *Review* (117) that Knight (118) from studies of the surface films of batyl, chimyl, and selachyl alcohols concluded that these alcohols were α-glyceryl ethers rather than β-glyceryl ethers. This view as to the structure of these alcohols has been confirmed recently. Adam (119) found by direct comparison of the surface films of natural batyl and chimyl alcohols with synthetic α-octyl- and α-hexadecyl-glyceryl ethers that they were identical. It has been shown by Davies, Heilbron & Jones (120) that oxidation of batyl alcohol with lead tetra-acetate gave formaldehyde and glycol

aldehyde octadecyl ether (m.p. 51°), which on oxidation with chromic acid gave glycolic acid octadecyl ether (m.p. 62–63°). Further studies on the glyceryl ethers and alcohols occurring in the unsaponifiable fraction of certain shark-liver oils and fish-liver oils have been published by André & Bloch (121) and by Weidemann (122).

Isomeric 9-10-dihydroxy octadecyl alcohols have been prepared by Collin & Hilditch (123) by oxidation of oleyl and elaidyl alcohols. When these alcohols are oxidized with hydrogen peroxide in acetic acid 9-10-dihydroxy octadecyl alcohols are obtained, which melt at 82° and 125–26° respectively. When oleyl phthalate is oxidized with alkaline permanganate and then hydrolyzed, 9-10-dihydroxy octadecyl alcohol (m.p. 126–27°) is obtained, whereas elaidyl phthalate under the same conditions yields the alcohol melting at 82°.

The normal primary alcohols from $C_{10}H_{21}OH$ to $C_{18}H_{37}OH$, the corresponding acetates and bromides, and also the corresponding acids and their ethyl esters have been prepared by Meyer & Reid (124). The melting-points of these compounds were determined accurately.

Several higher alcohols, *n*-tetracosanol and *n*-hexacosanol, have been isolated from spinach fat by Heyl & Larson (125).

A higher alcohol of unknown constitution named kahweol has been isolated by Bengis & Anderson (126) from the unsaponifiable fraction of coffee-bean oil. Kahweol crystallizes beautifully but the crystal form varies with the solvent. The substance is highly unsaturated and is exceedingly sensitive to heat, light, acids, and to oxidation. The composition corresponds to the formula $C_{19}H_{26}O_3$ and it contains one hydroxyl group. It melts at 143–143.5°; $[\alpha]_D$ — 204.5°. On catalytic reduction kahweol absorbs 6 atoms of hydrogen giving a stable compound

$$C_{19}H_{32}O_3 \text{ (m.p. 173–174°; } [\alpha]_D - 67.8°).$$

In the work already referred to by Piper, Chibnall & others (27) is recorded the synthesis of several higher secondary alcohols which may possess possible biological significance. The alcohols were prepared by the reduction of the corresponding ketones. The following new compounds were described: 15-nonacosanol (m.p. 83.6–83.8°); 14-nonacosanol (m.p. 79–79.3°); 12-nonacosanol (m.p. 74–74.5°); 10-nonacosanol (m.p. 74.8–75°); and 9-nonacosanol (m.p. 75.3–75.6°).

Some interesting data have been published on the melting- and freezing-points of mixtures of fatty acids and certain higher alcohols. Characteristic solidification points of fats in various solvents have been described by Lustig, Nabih & Wirnitzer (127) while Jennings (128) has determined the titre points of mixtures of lauric and myristic, lauric and palmitic, oleic and palmitic, oleic and lauric, and oleic and myristic acids. In the paper mentioned previously by Meyer & Reid (124) consideration was given to isomorphism and alternation in the melting-points of the compounds prepared by these authors such as alcohols, acids, etc., from C_{10} to C_{18}. Carey & Smith (129) studied systems composed of ethyl palmitate, ethyl margarate, ethyl stearate, and hepta- and octadecyl alcohols. Shriner, Fulton & Burks (130) have studied the ternary system composed of palmitic, margaric, and stearic acids. The results obtained would indicate that compound formation does not occur with margaric, palmitic, and stearic acids such as is formed with palmitic and stearic acids.

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THE CHEMISTRY OF THE LIPINS*

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The Phospholipins

The nature of the phospholipin fatty acids prepared from liver and from brain has been further elucidated. Klenk & Schoenebeck (1) have isolated from the phospholipins of ox liver a small amount of lignoceric acid; stearic was the predominating saturated acid, whereas, in the neutral fat, palmitic acid was present in larger quantity. Snider & Bloor (2) determined the proportion of stearic acid to palmitic acid in the lecithin derived from liver and found it to be 70:30. Klenk & Schoenebeck found evidence of the existence of oleic and linolic acid among the unsaturated acids as well as of arachidonic and clupadonic acids, the proportions of the last two acids being much higher than in the neutral fat, which more closely resembled the depot fat. Snider & Bloor found linolic in the acids both from the depot fat and from the phospholipins, preponderating however in the latter. In fish and in amphibia, Klenk found palmitic as the predominating saturated acid of the liver lipins. In the small shark, *Etmopterus spinax*, arachidonic and clupadonic were the chief unsaturated acids (3); estimation of the choline indicated that 45 per cent of the phospholipin present was lecithin. In the liver oil from *Rana temporaria* the C_{18} unsaturated acids with one or two double bonds were present, the more highly unsaturated acids belonging to the C_{20} and C_{22} series (4). Not only in mammals but in fishes and in amphibia the highly unsaturated acids of the liver are those containing 20 and 22 carbon atoms.

In the ether-soluble phospholipins of the brain, Klenk (5) identified in addition to oleic acid, gadoleic acid ($C_{20}H_{38}O_2$, m.p. 20°)—probably identical with that described by Bull (6) in codliver oil—arachidonic and docosatetrenoic acids and obtained evidence of the presence of yet more highly unsaturated derivatives containing 22 carbon atoms. Brown (7) working with ox brain found that docosatetrenoic and docosapentenoic acids predominated, and obtained some indication of the presence of a tetracosapentenoic acid. Page & Rudy (8) from the kephalin of human brain obtained octobromides of unsaturated acids yielding on reduction behenic and

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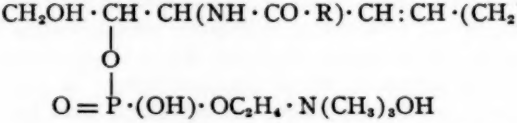
possibly arachidic acids. These octobromo derivatives of behenic acid occurred in soluble low-melting and in insoluble high-melting forms, a mixture of isomeric bromides of varying melting-points being present [Rudy (9)]. In the mother liquors from which Page & Rudy (8) had separated brain kephalin, stearic, the only saturated acid identified, formed 30 per cent of the total fatty acids; unsaturated C_{22} acids containing four and five double bonds formed 22 per cent of the total and C_{18} unsaturated acids accounted for another 22 per cent. Unsaturated acids containing 20 carbon atoms were not identified with certainty.

The changes which take place in kephalin when it is left exposed to the air were studied by Bülow & Page (10), who failed to establish a hydrolytic splitting of the fatty acids but found an increased proportion of hydroxyacids.

The Sphingomyelins

Probable structure.—



Klenk (11), after purifying the sphingomyelin derived from heart muscle, satisfactorily identified lignoceric and stearic acids among its hydrolytic products and showed that heart sphingomyelin closely resembled that obtained from brain. With water, both products formed milky solutions. Klenk pointed out that the widespread occurrence in the tissues of small quantities of the galactolipins and of sphingomyelin provided a source of lignoceric acid from which the C_{20} and C_{22} acids characteristic of lecithin and kephalin might be derived by oxidation.

From the phospholipin fraction of ox liver Klenk & Schoenebeck (1) separated a product resembling sphingomyelin, melting at 156° to 157° C, and yielding on hydrolysis sphingosine and lignoceric acid. Working with pig's liver, Fraenkel, Bielschowsky & Thannhauser (12) isolated a hygroscopic diaminophospholipin differing somewhat in its properties from the sphingomyelin derived from the brain. Its hydrolytic products were choline, sphingosine, and phosphoric acid and the three fatty acids, palmitic, stearic, and lignoceric, present in equivalent quantities. The presence of a group of sphingomyelins

differing in the nature of the fatty acid contained has already been indicated by Merz (13). The occurrence of these three fatty acids in equivalent quantities has led Fraenkel and his colleagues to suggest that the sphingomyelins are built up into salt-like complexes formed by the condensation of three sphingomyelin molecules with elimination of water between the choline and phosphoric-acid groups of adjacent molecules.

These authors suggest that the existence of such long-chain polydiaminophospholipins might be of great importance in producing the properties of irritability and conductivity characteristic of nerve tissue.

It is interesting in this connection to recall the structure of nucleic acid in which four complex nucleotides differing only in the nature of their bases are linked together, but in this case condensation takes place between the phosphoric-acid group and a pentose hydroxyl of adjacent molecules.

The Galactolipins

Klenk & Diebold (14) prepared pure cerebronic acid from brain and showed conclusively that the acid formed from it by oxidation with potassium permanganate was tricosanic acid; the presence of lower homologues was also indicated. Pure lignoceric acid (m.p. 84·5–85°) derived from brain must therefore be accepted as tetracosanic acid.

Sulphur-containing galactolipin.—The presence of a sulphur-containing galactolipin in brain was first observed by Thudichum. Blix (15) confirmed its existence in human brain probably as a potassium salt; he obtained from it on hydrolysis sulphuric and cerebronic acids, sphingosine, and galactose. Blix considered that probably from 20 to 25 per cent of the brain galactolipins occurred as sulphuric esters.

The preparation of the lipins.—Escher (16) has described the preparation from brain of a mixture of lipins in a fresh condition, white and with almost neutral reaction. After dehydration with alcohol or acetone, the extraction was carried out with a mixture of alcohol and light petroleum at 37°.

Separation of lecithin and kephalin.—Extraction of the lecithin cadmium chloride compound with a mixture of ethanol with either benzene or chloroform has been recommended for the preparation of lecithin free from kephalin [Pertzoff & Aisner (17)].

Physical properties.—Jones & Lewis (18), who investigated the capillary activity of lecithin-cholesterol dispersions, found that the reduction in surface tension of a lecithin dispersion compared with water was decreased or abolished by filtration. The clear filtrate had a surface tension only slightly below that of water so that the capillary effect could be ascribed to the large units in the dispersion which gave rise to the turbidity. Magistris (19) found that the diffusion of acids and alkalies into gelatine and agar gels was checked by lecithin and accelerated by cholesterol. The structure and properties of the liquid crystals formed by the phospho- and galactolipins have been described by Rinne (20).

Estimation.—A micro-method in which the phospholipin was precipitated with the protein by trichloracetic acid and extracted from the precipitate was described by Norberg & Teorell (21); other modifications in the determination of lipin phosphorus have been recorded by Man & Peters (22), Karrsen & van Wering (23), and by Milbradt (24).

The Function of the Phospholipins

Klenk & Schoenebeck (1) have pointed out that, if in the liver the fatty acids are built up into phospholipins and there desaturated, a hypothesis put forward by Leathes largely on the ground of the high-iodine values of the liver phospholipin acids, the process cannot be a simple one, since it is not in the C_{18} acids, but in the C_{20} and C_{22} acids, which are absent from the depot fats, that desaturation occurs; it is only in plants that high desaturation takes place in the C_{18} acids.

Considerable attention has been paid to the effect of diet on the amount of tissue phospholipins, especially those of the blood and liver. Bloor (25) has published an interesting discussion on the influence of diet on the blood lipins. Very great variations have been found in the phospholipin content of the plasma; for instance, in two groups of healthy dogs, examined in Bloor's laboratory, at an interval of four years, the average plasma phospholipid percentages of the two groups were respectively 400 and 70 mg. Continuous feeding of diets alternating between one relatively high in fat and one relatively low produced definite changes in the level of phospholipin and cholesterol in the plasma of dogs and rabbits, a higher level of both substances always following the high-fat diet; the variations were much more marked in the rabbit than in the dog. This difference in phospholipin level would be consistent with their function

as substances aiding either in the transport or in the metabolism of fat. Increases in the amounts of plasma phospholipins following the ingestion of fat were also recorded by Wendt (26) and by Man & Gildea (27). The latter authors observed that the ingestion of large amounts of fat produced in humans a marked rise in the serum fatty acids and a less marked increase in the serum phospholipins. A meal containing carbohydrate and protein with a moderate amount of fat was followed by a rise in the iodine value of the serum fatty acids but not in that of the acids from the phospholipin fraction. Artom (28) recorded a marked increase in the liver phospholipins up to the seventh hour after feeding dogs with neutral fats and then a gradual return to the initial value. He regarded this as evidence that the phospholipins were intermediate substances in the metabolism of fat. Pasternak & Page (29) injected moderate amounts of kephalin into the blood stream and found it quickly absorbed; after large injections the level of the plasma phospholipin remained higher for some time and was accompanied by an increase of the liver phospholipin.

Artom (30) injected an emulsion of iodised fat into the external jugular vein of dogs and after the lapse of a certain time estimated the iodine in the fatty acids of the blood, the liver, and other tissues. In the liver, from one to five hours after the injection, a large proportion of the iodised fat was present but only a very small proportion in the phospholipin fraction. The iodine in the phospholipin of the blood corpuscles formed a considerable proportion of the total iodine. Much less was contained in the plasma, and of this only a small proportion was present in the phospholipin fraction. Artom concluded that a marked synthesis of phospholipin took place at the expense of the fatty acid injected, but that evidence was lacking as to whether the phospholipin was synthesised in the corpuscles as suggested by Bloor (31) or whether it was elaborated elsewhere and the corpuscles utilised for its transference. Boyd (32), who had found the phospholipin content of the leucocytes in dogs was 710 mg. per 100 gm., observed that in a group of young women the value was 802 mg. per 100 gm. (33); that of the plasma in dogs was 53.7 mg. per 100 cc., an unusually low value. The phospholipin acids were found to be much more unsaturated than the total fatty acids derived from blood. Cuthbertson (34) found that the fatty acids from the phospholipin fraction of human skeletal muscle and from the triglycerides of the subcutaneous fat were very similar in composition, but that the acids from the neutral fat of skeletal muscle contained a

smaller proportion of carbon and a higher proportion of oxygen. According to Okey & Stewart (35) the level of the lipins in the blood was affected by menstruation. Interesting results have been brought forward by Schaible (36), with regard to the differences in the amounts of lipins in the blood of lactating and non-lactating animals. The average fatty-acid content of the plasma phospholipins of the lactating animals was 68.9 mg. per 100 cc. and that of the plasma from the non-lactating group reached the extraordinarily low value of 10.5 mg. The iodine number of the phospholipin fatty acids was, however, lower in the lactating group. This large difference in the fatty-acid percentages found in the two groups, if of general occurrence, would be of especial interest in view of the work of Meigs, Blatherwick & Cary (37), which suggested that milk fatty acids were liberated from the phospholipins of the blood as it circulated through the mammary gland. In view of Bloor's findings on dogs, a large number of determinations would have to be made before it would be possible to draw definite conclusions and the composition of the diet would have to be taken into account. The addition of phospholipin to the diet did not appear to increase the phospholipin content of the milk [Trantmann & Kirchof (38)].

Many experiments have been carried out in order to determine the effect of diet on the nature of the phospholipin unsaturated acids. Sinclair (39) argued that if it be a function of the phospholipins to transport the fats to the tissues, there to serve as fuel, there must be a rapid turnover of the phospholipin fatty acids. He observed that, when rats are transferred from a fat-free or fat-poor diet to one containing 20 per cent of cod-liver oil, the iodine value of the phospholipin fatty acids rose in two days from 110 to 135 and then more slowly to 160. On the reverse change being carried out, the high iodine values persisted for a long time after the change of diet. A marked increase of weight accompanied the inclusion of cod-liver oil in the diet. The iodine values were not affected by keeping the rats at low temperatures when the intensity of the fat metabolism would presumably be increased.

Sinclair (40) also found that when rats were fed on certain distinctive diets, the relative amounts of saturated to unsaturated acids in the phospholipins from the muscles and viscera remained constant at 31 to 69, although the degree of unsaturation might be much raised, whereas in the neutral fat increase in unsaturation was accompanied by a decrease in the proportion of saturated acid present. When rats

were raised on a synthetic diet he found (41) that the addition of small amounts of cod-liver oil resulted in a great increase in the iodine number of the phospholipin acids, there being a definite quantitative relationship between the amount of cod-liver oil ingested per day and the iodine value of the phospholipin fatty acids. The liver phospholipins were much more influenced than those of the skeletal muscles. Lard and to a less extent cocoanut oil produced results similar to those obtained with cod-liver oil. The small amounts of fat which were sufficient to produce a marked increase in the degree of unsaturation of the phospholipins produced no apparent effect on the degree of unsaturation of the neutral fat. Sinclair suggested the possibility of a causal relationship between the low degree of unsaturation of the tissue phospholipins and the subnormal growth of rats on fat-free diets.

Monaghan (42) observed that starvation diets deficient in vitamins A and B show a lower proportion of liver phospholipins to the body weight than in the control animals.

Best & Hershey (43) kept dogs from which the pancreas had been completely removed on a daily diet of 300 gr. of lean beef muscle and 100 gr. of sucrose, the insulin dosage being regulated to allow a slight glycosuria. After some months, symptoms developed which the authors believed to be due to defective liver function and which were relieved by the addition of lecithin to the diet. These characteristic symptoms were associated with fatty infiltration and degeneration of the liver, in some cases the sugar excretion being greatly diminished. The onset of the condition was accelerated by the addition of fat in the diet. When crude lecithin was added to the diet, the symptoms disappeared and the output of sugar was markedly increased, the urinary nitrogen being very slightly affected. In one case, after the addition of lecithin to the diet, the sugar output was raised from 6 to 17 gm. daily, an amount for which the authors found it difficult to account unless it had its source in some constituent of the lecithin. The fat feeding appeared to have produced changes in the liver which inhibited gluconeogenesis, but which disappeared under the influence of some constituent of the lecithin. Best, Hershey & Huntsman (44) then investigated the effect on normal rats which had been receiving for three weeks a daily ration containing 2.5 gm. of fat (iodine number, 40); the livers contained large amounts of fatty acids (iodine number, 100). This increase of fat was not shown in the animals which had received lecithin as well as

fat in the diet; the liver fat of these animals was more unsaturated (iodine number, 132). Subsequently Best, Ferguson & Hershey (45) have found that the effect of lecithin in depancreatised animals may be obtained by the addition of choline to the diet and that a high-fatty diet accompanied by choline is well tolerated. Since aminoethyl alcohol showed no similar action, kephalin could not replace lecithin.

Jost & Sorg (46) found that in artificially incubated eggs the unsaturated content of the neutral fats showed little change, while the fatty acids of the phospholipins lost, to a great extent, their high degree of unsaturation. Nearly half the phospholipin present in the egg before incubation was used up during development. At the end of development, the phospholipin of the embryo had a high iodine value, that of the yolk being small.

The deduction by Jost (47) from the experiments on liver perfusion that lipin fatty acids were converted into sugar has been criticised by Page & Young (48) on the ground that the small increase of sugar observed in the perfusion liquid might arise from the glycerol contained in the 5 to 8 gm. of kephalin added. They found that the sugar output was not increased by injecting an aqueous emulsion of brain phosphatide into phlorhizinised dogs, the D/N ratio not being appreciably altered.

Rondoni (49) concluded that the phospholipins exerted a specific influence on the tryptic enzymes, lecithin inhibiting the action of proteinase and carboxypolypeptidase, kephalin only that of the proteinase. Inhibition is produced only during the action of enterokinase, the enzyme when already activated being unaffected by lecithin.

Phospholipins in plants.—Buchan Smith & Chibnall (50) have isolated kephalin and lecithin from the forage grass, cocksfoot, and have identified in them linolic and linolenic acids, the proportion of saturated acid being definitely less than that of the unsaturated ones. From carrots, Bleyer & Diemair (51) have obtained a dialysable substance with a N/P ratio of 1.

Phospholipins in bacteria.—Anderson and his colleagues have shown that other members of the acid-fast group of bacteria contain complex carbohydrate-containing phospholipins similar to those isolated from the tubercle bacillus. In *Bacillus leprae* (52), the substance isolated contains palmitic acid, tuberculostearic acid, and unsaturated acids giving palmitic and stearic acids on reduction. In Timothy bacillus, palmitic and an inactive liquid-saturated acid similar to phthioic acid were present (53).

Changes in the lipin content of the tissues in disease.—Very large increases in the amount of phospholipin in the liver and in the spleen have again been recorded by Epstein & Lorenz (54) in Neumann-Pick disease: the cholesterol content of the liver, unlike that of the spleen, was much increased. In cancerous tissue, a high content of both cholesterol and phospholipin has been recorded by Bierich, Detzel & Lang (55). De Voss (56) found the average value for phospholipin in the sera from 61 cases of cancer was 150.5 mg. per 100 cc., as compared with an average value of 178 mg. per 100 cc. from 34 pathological non-cancerous cases. Uramoto (57) observed that during the growth of Flexner-Jobling rat carcinoma there is an increase in the combined cholesterol and inorganic phosphorus, a decrease in the lipoid acid-soluble organic and in protein phosphorus. Willheim & Stern (58) recorded that cancer sera unlike normal sera have lost the power to dissolve lipins from cancer cells. An increase in the amount of fatty acids and phospholipins in the blood was found by Ohata (59) in experimental scurvy in the blood of guinea pigs. Rosen, Krasnow & Lyons (60) found that cholesterol and lecithin values both tended to be high in skin diseases and in syphilis; in cryptogenic epilepsy normal values were recorded.

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THE CHEMISTRY OF THE STEROLS, BILE ACIDS, AND OTHER CYCLIC CONSTITUENTS OF NATURAL FATS AND OILS*

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Our conceptions of the constitution of sterols and bile acids have undergone such drastic and far-reaching changes since the subject was last reviewed[1] that it seems desirable to give an outline of cholane chemistry as well as a more detailed review of work published during the period in question. Such a course is all the more indicated since those who are not intimately acquainted with the literature of one of the most complex chapters of organic chemistry will find the difficulties of adjusting the old to the new formulae almost bewildering, made all the more so by a perplexing, and occasionally illogical, nomenclature.

When Wieland (1) and Windaus (2) reviewed in the *Nobel Lectures of 1928* the results of their pioneering investigations on the structure of the bile acids and sterols, it seemed as if the last chapter in the story of one of the most brilliant researches of organic chemistry had been written. The ring system of an important group of natural substances had been established with a degree of certainty which seemed to be final, and it only remained to assign suitable positions to two of the carbon atoms. In the formula of cholic acid (I) (see page 88) these were provisionally accommodated as a C_2H_5 group attached to C_{10} in ring IV.

The task of placing the two "homeless" carbon atoms in the molecule was subsequently undertaken by Wieland and his collaborators. It soon became apparent that the C_2H_5 group was not in ring IV, and in spite of four years' systematic effort it proved to be impossible to place the two carbon atoms elsewhere in the ring system. The old formula became thus untenable. Some of the many ingenious attempts of Wieland to arrive at a solution of the problem were described by Windaus in the previous survey of the subject.[2]

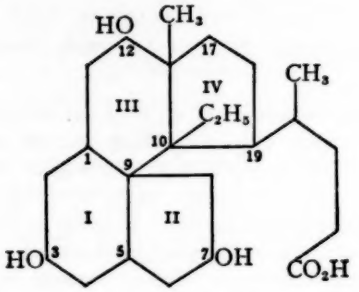
In the same year a fundamentally new structure for the cholane

* Received January 30, 1934.

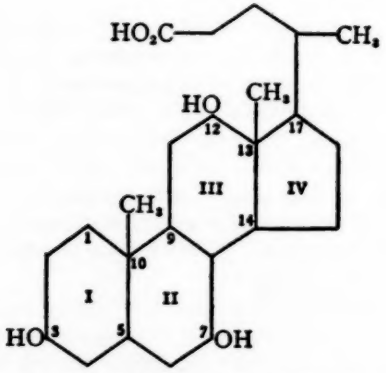
[1] *Ann. Rev. Biochem.*, **1**, 109 (1932).

[2] *Ibid.*

ring system was advanced by Rosenheim & King (3) and was at once accepted by Wieland and Windaus as offering a satisfactory explanation of the difficulties encountered hitherto.



I. Wieland, 1928



II. Rosenheim & King, 1932; Wieland & Dane, 1932

In their proposal of the new formula, Rosenheim & King based their conclusions mainly on certain experimental facts which had not received the attention they deserved. Thus Bernal (4), in the course of an X-ray crystallographic examination of calciferol (crystalline vitamin D), made the incidental observation that the dimensions of the ergosterol molecule and the position of the side chain in the accepted sterol formula could not be reconciled with the experimentally determined length of the molecule. Adam & Rosenheim (5) had

previously also obtained results which were "nearly impossible to interpret" in the light of the accepted formulae, when investigating the surface films of sterols.

The clue to the solution of the problem was afforded by a reconsideration of the work of Diels and his collaborators (6). By dehydrogenation of cholesterol and cholic acid with palladium-charcoal or selenium, Diels succeeded in isolating the fully aromatic hydrocarbon chrysene, $C_{18}H_{12}$. Cholesterol and ergosterol yielded with selenium, in addition, the two hydrocarbons $C_{18}H_{16}$ and $C_{25}H_{24}$. The significance of this important discovery was not recognised, either at that time or later, owing to the implicit acceptance of the sterol formula then in use. The isolation of chrysene in particular was held by Diels & Karstens (7) to be without value for the discussion of the cholic acid formula [cf. also (8)].

The new cholane formula, primarily based on the formation of chrysene in Se-dehydrogenation, consists essentially of a reduced phenanthrene ring system, to which ring IV of the old formula is attached as a *cyclo*pentane ring, carrying the side chain at C_{17} instead of at C_{19}. This formula (II) satisfies the requirements of the X-ray measurements [Bernal (9)] and is in complete accord with all the experimental facts accumulated during the last thirty years. Most of the material available in the older literature has been adjusted to, and reconciled with, the new formula in an exhaustive article by Windaus (10), to which the reader is referred for details. Recent additional evidence will be reviewed subsequently.

The change in the conception of the constitution of the sterols and bile acids has already acted as a stimulus to the work in other fields of biochemistry. As foreseen by its authors, the new cholane formula was clearly applicable to the ring-system of the oestrogenic hormones and its immediate adoption by Marrian (11) and Butenandt (12) clarified the views as to the constitution of the various members of this group. Further, the somewhat unexpected fact now emerges that an apparently unconnected series of physiologically highly active substances, such as calciferol (crystalline vitamin D), oestrogenic hormones, carcinogenic hydrocarbons, cardiac glucosides (strophanthin, etc.), and probably also toad poison (bufotoxin), have a phenanthrene nucleus in common.

Nomenclature.—Recent progress in cholane chemistry seems to call for the removal of certain anomalies of nomenclature, which have accumulated during the many years that have passed since

Mauthner's researches, begun in 1893, laid the foundation of modern sterol chemistry. It so happens that names which were given haphazard to derivatives suggest a non-existing relationship, whilst others fail to express a relation now known to exist. Striking examples are *allo*cholesterol and *allo*cholanic acid, which are not related to each other, whilst the now obvious connection (see later) between *allo*cholesterol, cholestenone, *pseudo*cholestene, and coprosterol finds no expression in their names. In this particular case a convenient solution is available by the systematic use of the prefix "copro" as in the following series:

Proposed Nomenclature	Old Nomenclature
Coprostanol (saturated alcohol)	Coprosterol
Coprostenol (unsaturated alcohol)	*Allo*cholesterol
Coprostanone (saturated ketone)	Coprostanone
Coprostenone (unsaturated ketone)	Cholestenone
Coprostane (saturated hydrocarbon)	*Pseudo*cholestane
Coprostene (unsaturated hydrocarbon)	Pseudocholestene

It is further advisable to restrict the use of the prefix *"allo"* to those bile-acid derivatives in which the steric configuration of rings I and II corresponds to *trans*decalin (i.e., *allo*cholanic acid, *allo*norcholanic acid, 3-hydroxy*allo*cholanic acid, etc.); the corresponding acids of the *cis*-series are cholanic, norcholanic, 3-hydroxycholanic acid, etc.

A further necessary change of nomenclature concerns the use of the term "pseudo," which denotes alternately a structural and a steric isomerism, as in *pseudo*cholesterol and *pseudo*coprosterol respectively. In this instance the difficulty is overcome by employing the prefix "epi-" in all those cases where the isomerism is due to the steric inversion of the OH group, as for instance at C_3 which, in this particular case, is connected with non-precipitability by digitonin (i.e., *epi*-coprostanol, *epi*-cholestanol, etc.).

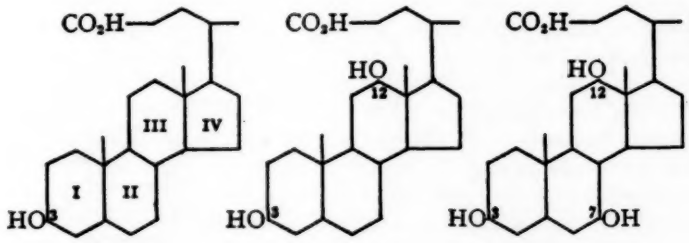
A remodeling of the picturesque but often perplexing bile-acid nomenclature, although feasible on the modern structural basis, is undesirable for the reason that interference with established historical usage is uncalled for as long as it is not misleading.

Constitution of the Bile Acids

Owing to the distribution of the hydroxyl groups in different rings, and the subsequent ease of attack, the bile acids yielded in-

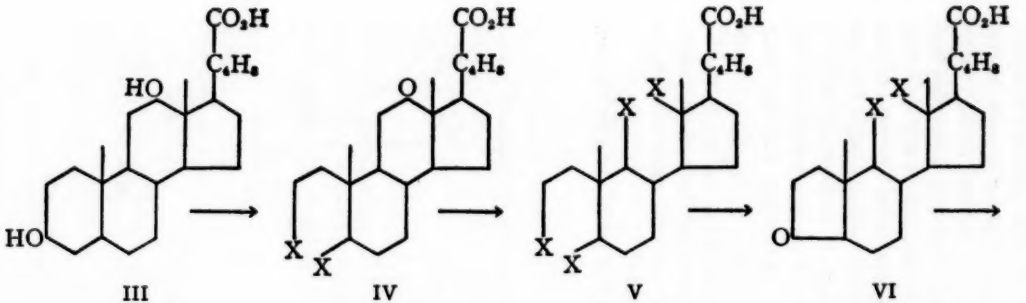
formation about the cholane ring system more rapidly than cholesterol with its single hydroxyl group in ring I. The positions assigned to the hydroxyl groups in the unconjugated bile acids have undergone an essential rearrangement, necessitated by the revised interpretation of previous experimental results. Avoiding a detailed and unprofitable discussion of past errors, it is sufficient to say that they arose from a single assumption, plausible enough at the time it was made, but clearly unjustified in the light of later knowledge, i.e., the placing of the distinctive hydroxyl group of deoxycholic acid in ring II, attached to ring I, and assumed to be a five-membered ring in reliance on Blanc's rule (13). This assumption, together with others (i.e., rupture of ring I between C_2 and C_3 in deoxybilianic acid) led to a formulation in which a quaternary carbon atom, C_9 of the old formula (I), was the point of fusion of three rings, resulting in a most unusual ring system which was the main obstacle to a rational interpretation of later degradations.

The new formulae of the best-known bile acids are given below, followed by a list of the others.



Lithocholic acid Deoxycholic acid Cholic acid

Acids	Position of OH Groups at C	Bile
1. *Mono-hydroxycholanic* $C_{24}H_{40}O_3$		
Lithocholic	3	Man, ox
2. *Di-hydroxycholanic* $C_{24}H_{40}O_4$		
a) Deoxycholic $C_{24}H_{40}O_4$	3, 12	Man, ox, sheep, etc.
b) Hyodeoxycholic $C_{24}H_{40}O_4$	3, 6	Pig
c) Chenodeoxycholic $C_{24}H_{40}O_4$	3, 7	Goose, hen, man
3. *Tri-hydroxycholanic* $C_{24}H_{40}O_5$		
a) Cholic $C_{24}H_{40}O_5$	3, 7, 12	Man, ox, sheep, etc.
b) β-Phocaecholic $C_{24}H_{40}O_5$	3, 7, 23	Walrus, seal

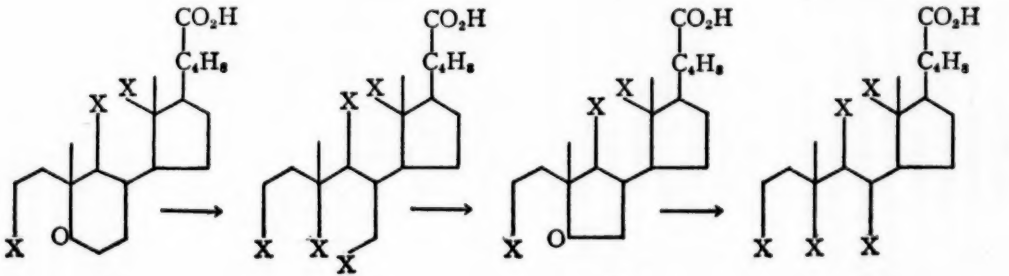


III
Deoxycholic-

IV
Deoxybilianic-

V
Choloidanic-

VI
Pyrocholoidanic-



VII
Prosolanellic-

VIII
Solanellic-

IX
Pyrosolanellic-

X
Norsolanellic- (Biloidanic) acid

X = CO_2H

An outline of the main degradations of these acids, which established the foregoing constitutional formulae, is as follows:

1. On dehydration by vacuum distillation they form unsaturated acids (cholenic, choladienic, cholatrienic acids), which on catalytic reduction are converted into one and the same monobasic cholanic acid $C_{24}H_{40}O_2$, the parent acid of the group. Hyodeoxycholic acid yields *allo*cholanic acid in addition. The relation of the bile acids to cholesterol was made clear by the fact that cholanic acid is obtained from coprostane (*pseudo*cholestane) and *allo*cholanic acid from cholestane by oxidation [loss of $\cdot CH(CH_3)_2$ from the side chain].

2. On oxidation with CrO_3 all the acids give the corresponding keto acids (monoketo-, diketo-, and triketo-cholanic acids), usually called "dehydro" acids, which yield on further oxidation, by rupture of ring I between C_3 and C_4, tricarboxylic acids called lithobilianic, deoxybilianic (IV), and bilianic acids respectively (in addition the isomeric *"iso"* acids are formed by ring rupture between C_2 and C_3).

3. When heated in a vacuum, lithobilianic and deoxybilianic acids lose CO_2 and H_2O, and give pyrolithobilianic and pyrodeoxybilianic acid. The same procedure leads to pyrocholoidanic acid, $C_{23}H_{32}O_6$ (VI), when applied to choloidanic acid, $C_{24}H_{36}O_{10}$ (V), a pentacarboxylic acid formed by oxidation of deoxybilianic acid (IV) with HNO_3 (rupture of ring III containing the carbonyl group). One of the chief reasons for discarding the old formula was its failure to afford a reasonable formulation of pyrocholoidanic acid.

4. Further oxidation of pyrocholoidanic acid leads through an intermediate stage to a hexabasic acid, $C_{23}H_{34}O_{12}$, in which only one ring (ring IV) is intact and which has therefore been called solanellic acid (*solus anellus*) (VIII). Pyrolysis of this acid yields pyrosolanellic acid (IX), the cleavage of which leads to the so-called biloidanic acid (X), $C_{22}H_{32}O_{12}$, which is also obtained from cholic acid ($\rightarrow$ bilianic $\rightarrow$ cilianic $\rightarrow$ ciloidanic $\rightarrow$ biloidanic acid). This acid forms a connecting link between deoxycholic and cholic acids.

5. On oxidation of pyrodeoxybilianic acid with potassium permanganate, the diketodibasic acid, $C_{23}H_{46}O_6$ (XV), is obtained, which passes through various stages into the important tribasic acid, $C_{13}H_{20}O_6$ (XVIII), containing a *cyclo*pentane ring.

6. Degradation of the side chain in stages (Grignard reaction followed by oxidation) proved the presence of the group $\cdot CH(CH_3)(CH_2)_2 \cdot COOH$, and led finally to the dibasic aetiobilianic acid,

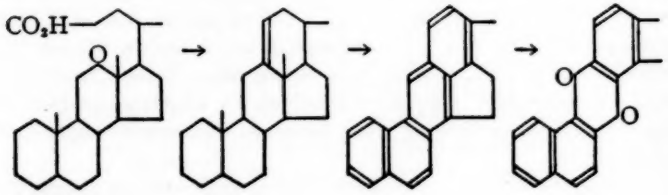
X = CO_2H

$C_{19}H_{30}O_4$. Pyrolysis of this acid yields an anhydride and characterises ring IV as a five-membered ring.

It is outside the scope of this review to reformulate these complex reactions, which represent only a part of the researches carried out by Wieland, Borsche, Schenk, and others since 1912. They are all easily interpreted by means of the new cholane formula, and one example only, that of pyrocholoidanic acid, leading through solanellic to biloidanic acid, is given on account of the important rôle which these acids played in the development of the constitutional formulae (see above).

Whilst it is obvious that the ring system of the new cholane formula affords an adequate interpretation of all the known bile acid reactions, it seemed desirable that the assumptions made with regard to the position of the distinctive hydroxyl group of deoxycholic acid and of the side chain should be experimentally verified. The position at C_{17} was assigned to the latter by Rosenheim & King (3), since it offered a natural explanation for the ease with which ring closure occurs (see XI and XII) between the side chain and ring III, in the derivatives of deoxycholic acids which contain a CO group at C_{12}. The presence of such a group implies a hydroxyl group at C_{12} in deoxycholic acid.

Evidence for the correctness of both these assumptions has now been furnished by the further study of the unsaturated hydrocarbon, dehydronorcholene (XII), which is obtained on pyrolysis of 12-ketocholanic acid (XI). Dehydronorcholene is dehydrogenated by selenium to the aromatic hydrocarbon, methylcholanthrene (XIII)

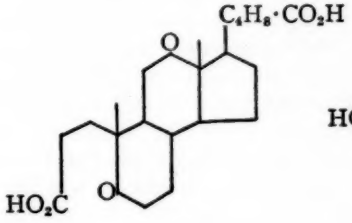


XI XII XIII XIV

(14). The constitution of the latter has been experimentally determined by Cook, Hewett & Haslewood (15) through its conversion into 5,6-dimethyl-1,2-benzanthraquinone (XIV), which furnished on oxidation, anthraquinone-1,2,5,6-tetracarboxylic acid, identical with

the acid formed by oxidation of 1,2,5,6-benzanthraquinone. Incidentally these changes support the five-membered structure of ring IV in the bile acids.

The allocation of positions to the two tertiary methyl groups at C_{10} and C_{13} is not definitely proved, but the indirect evidence in its favour is accumulative and almost conclusive. The presence of a methyl group at C_{10} follows (*a*) from the isolation of α-methylglutaric acid, $CO_2H \cdot CH(CH_3) \cdot CH_2 \cdot CH_2 \cdot CO_2H$, via *n*-butanetricarboxylic acid on oxidation of a diketodicarboxylic acid, $C_{23}H_{34}O_6$ (XV), obtained from pyrodeoxybilianic acid; (*b*) from the stepwise Grignard degradation of ring I in cholesterol by Tschesche (16) to a monobasic acid (XVI) which has a tertiary methyl adjacent to the carboxyl, since the acid is very difficult to esterify; (*c*) from the observation of Windaus that the dicarboxylic acid, $C_{22}H_{38}O_4$ (XVII), obtained by ultimate degradation of rings I and II in choles-



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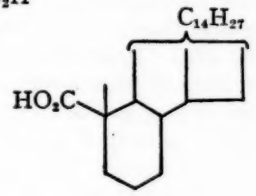
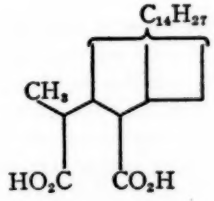
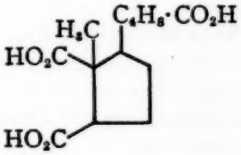


XVI



XVII



XVIII

terol, forms an anhydride and not a cyclic ketone and must therefore contain a branched chain with a methyl group; the latter can only have been at C_{10} [Wieland & Dane (17)].

The allocation of a tertiary methyl group at C_{13} follows from

the properties of the acid, $C_{13}H_{20}O_6$ (XVIII), containing the fourth ring of the bile acids intact and obtained both from deoxycholic acid (III) and from 12-ketocholanic acid (XI) and is further supported by the identification of Diels's hydrocarbon, $C_{18}H_{16}$, obtained by Se-dehydrogenation of cholic acid, as 3-methyl*cyclo*pentenophenanthrene [Kon (18); see later].

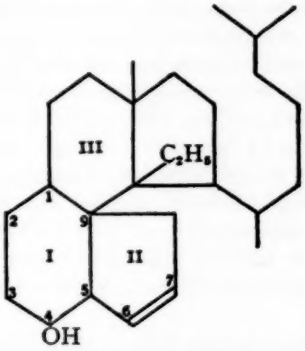
One method adopted by Wieland and his co-workers for the controlled degradation of the cholane structure has been to study the oxidative ring rupture of mono-hydroxycholanic acids containing the hydroxyl group, and hence the vulnerable point, in different rings. Lithobilianic acid, the tricarboxylic acid from 3-hydroxycholanic acid (lithocholic acid), gave on pyrolysis a ketonic acid, arising from the six-membered ring I. The second of the three possible acids of the bilianic type, *neo*-lithobilianic acid (13, 19) was obtained from 12-ketocholanic acid and gave rise to an anhydride on pyrolysis, a fact which, in reliance on Blanc's rule, was interpreted as evidence for a five-membered ring. The last of the three isomeric acids, thilobilianic acid from 7-hydroxycholanic acid, has been investigated only recently (20). Pyrolysis of this acid again yielded an anhydride. On the basis of Blanc's rule, which has been used systematically as an index of ring size in the cholane group (1), it followed logically that ring II as well as ring III should be five-membered, a conclusion which is irreconcilable with previous results. It was the recognition of the impossibility of such a conclusion which induced Wieland & Dane (20) to reject the cholane formula hitherto employed and to adopt the new formulation of Rosenheim & King, which had just then become available.

It is now evident that Blanc's rule must allow of anhydride formation even from 1,6-dicarboxylic acids, if the new ring formed is situated between the cyclic systems. This exception to Blanc's rule, hitherto overlooked, does not, however, invalidate the general rule of ketone formation from 1,6-dicarboxylic acids, since the classical method (pyrolysis of Ba salts) leads to a cyclic ketone even in those exceptional cases (Stange, 21), where Blanc's modification of the old method (distillation with acetic anhydride) yields an anhydride.

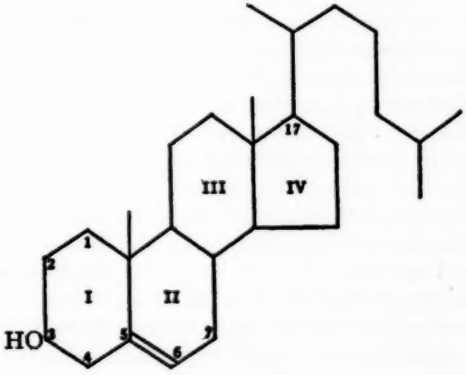
Only one uncertain point in bile-acid chemistry remains now to be cleared up, viz., the position of the double bond in apocholic and dihydroxycholenic acids. This subject as well as the related question of the isomeric cholatrienic acids [Shimizu *et al.* (22)] requires further experimental work before it can be profitably reviewed.

The Constitution of Cholesterol

From the formulae below it will be seen that the essential change made in the new formula concerns the ring system, in which the five-membered ring II, of the old formula, is converted into a six-membered ring and attached to the other side of ring I.



Old formula (1928)



New formula (1932)

This change necessitated a transference of the hydroxyl group from C_4 to C_3, a change long overdue on biogenetic grounds and one which emphasises the common origin of cholesterol and the bile acids, already demonstrated by interconversion of simple derivatives.

The new position of the hydroxyl group led in logical sequence to a transference of the double bond from $C_8 : C_7$ to $C_5 : C_6$. The side chain is now attached to C_{17} as in the bile acids.

The available mass of earlier observations is convincingly in favour of the new formula [see Windaus (10)] and has been supplemented by more recent work, which will now be reviewed.

The identity of the ring systems of cholesterol and the bile acids, already proved by the reaction: cholesterol → coprostane (ψ-cholestane) → cholanic acid, was further substantiated by the step-wise degradation of lithocholic acid (3-hydroxycholanic acid) into two acids, $C_{22}H_{34}O_6$ and $C_{21}H_{32}O_6$ (23), identical with those previously obtained by Windaus by degradation of rings I and II of cholesterol.

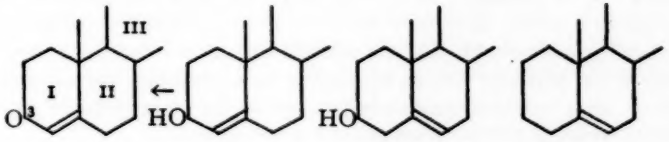
Starting in the reverse direction, Stange (24) converted cholesterol into 6-keto*allo*cholanic acid by the stages: 6-chlorocholestane → 6-chloro*allo*cholanic acid → 6-hydroxy*allo*cholanic acid → 6-keto*allo*cholanic acid and thus arrived at an acid identical with that previously obtained by Wieland & Dane (25) from hyodeoxycholic acid.

Incidentally these changes demonstrate the incorrectness of an earlier deduction which allocated the distinctive hydroxyl group of hyodeoxycholic acid to ring III.

The new cholane structure allows for the formation of only two ketones in ring II, whereas Windaus had described three ketones, viz., cholestane-6-, -7-, and -8-one in ring II of the old formula. From the method of their preparation it follows that in the new formula the two latter are cholestane-6- and -7-one respectively, whilst the ketone group of cholestane-6-one (of the old structure) is, according to Tschesche (26), actually in ring I of the new formula. On oxidation this ketone, now cholestane-4-one, yields a dicarboxylic acid identical with dihydro-Diels acid (see p. 101). It is of interest to note that this acid on pyrolysis gives either an anhydride or a ketone, according to the temperature at which the reaction is carried out.

The position of the hydroxyl group at C_3 follows from the conversion of hyodeoxycholic acid (3,6-dihydroxycholanic acid) into 3-hydroxy-6-ketocholanic acid, which by reduction of its semicarbazone gave among other products 3-hydroxy*allo*cholanic acid (25), identical with the acid obtained from cholesterol by the stages 3-chlorocholestane → 3-chloro*allo*cholanic acid → 3-hydroxy*allo*cholanic acid. This degradation supplied incidentally a further proof of the identity of the ring systems of cholesterol and the bile acids.

The position of the double bond at C_5: C_6 in cholesterol and certain substances closely allied to it has been established by a number of independent observations. Menschick, Page & Bossert (27), on the basis of measurements of absorption spectra, showed that coprostenone (cholestenone) was an α:β-unsaturated ketone. As was first pointed out by Miss Dane, the formulation of its oxidation products necessitated the removal of the keto group from the 4-position previously assigned to it, to the 3-position in ring I (XIX). It was further demonstrated by Bonstedt (28) that coprostenone is obtained from coprostenol (*allo*cholesterol) by oxidation with chro-



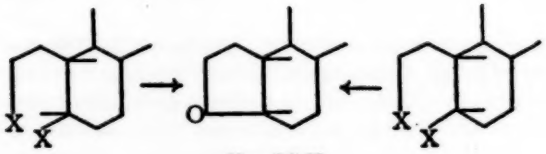
XIX XX XXI XXII

mic acid, so that in coprostenol (XX) the double bond is also α:β- to the hydroxyl group. Furthermore Lettré (29) later was able to convert coprostenone (XIX) into coprostene (ψ-cholestene) by a Kishner-Wolff reduction of the semicarbazone. Since on catalytic reduction coprostene is convertible into coprostane, whilst coprostenol passes into coprostanol (coprosterol), the clearly established relationship of these substances seems to justify the change in nomenclature proposed on page 90.

The position of the double bond between C_4 and C_5 is thus the same in coprostene, coprostenone, and coprostenol. On the other hand, it had long ago been established by Windaus that cholesterol is a β:γ unsaturated alcohol in which the hydroxyl group and the double bond are in different rings. The structure (XXI) must therefore now be accorded to cholesterol and (XXII) to cholestene. This formulation allows the various changes undergone by cholesterol to be explained in a simple manner. It involves the justifiable assumption of a movement of the double bond to the α:β position during the formation of coprostenone from cholesterol, which is strictly analogous to the easily formulated movement occurring during the isomerisation (by means of HCl) of cholesterol and cholestene, into coprostenol and coprostene respectively.

The stereochemistry of the ring systems of the sterols and bile

acids is a subject which still awaits full development. The X-ray measurements of Bernal on the sterols demand a fairly flat structure, which is only possible if the middle rings II and III are joined in a *trans-* position [Ruzicka *et al.* (30); cf. Bernal in "Discussion on Oestrin" (58)]. On the stereochemical relationships of rings I and II there is a considerable body of experimental evidence which all points in one direction. Windaus and his pupils have made a study of the ketones formed on pyrolysis of the dicarboxylic acids obtained on opening ring I either between C_2 and C_3 or between C_3 and C_4, both in the sterols and in the bile acids. The principle on which deductions are made may be expressed as follows: when the pyrolytic cyclisation of a 1,6-dicarboxylic acid leads to the formation of a five-membered ketone-ring attached to a six-membered ring, then in all cases where one of the carboxyl groups is attached to a secondary carbon atom of the ring, a rearrangement is possible to the form with the least strain which is a bicyclic ketone of the *cis*-series.

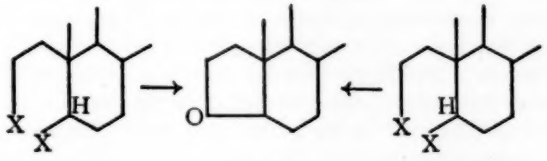


$X = CO_2H$

The results obtained both in the sterols and in the bile acids show that rings I and II are *trans* in cholestane and its derivatives and *cis* in coprostane and its derivatives. Ruzicka (30) arrives at the same conclusion from a comparison of the densities, melting points, and refractive indices of cholestane and coprostane with those of *trans-* and *cis-* decalins. The only difference therefore between cholestane and coprostane lies in the spatial orientation of the hydrogen atom on C_5.

That rings III and IV are also attached in a *trans-* position is deduced by Wieland & Dane (17), who found that the tricarboxylic acid, $C_{13}H_{20}O_6$ (XVIII), derived from the bile acids and containing only ring IV, yields an anhydride which on hydrolysis gives a stereoisomeric acid. Since the anhydride contains a five-membered ring attached to another five-membered ring, considerations of ring strain demand the representation of the process as a change from a *trans-*dicarboxylic acid to a *cis*-dicarboxylic acid.

It is important to note that the stereochemical arrangement around C_5 determines the manner in which ring I is opened when an oxygen atom is at C_3. In the cholestane series oxidative fission of ring I occurs between C_2 and C_3, but in the coprostane series mainly between C_3 and C_4. In this connection it is of interest to mention the difficulty which arose in interpreting, by means of the new sterol formula, the reported formation (Windaus & Rieman) of *iso*-lithobilianic acid from coprostanol by drastic oxidation [fissure of ring I and loss of $\cdot CH(CH_3)_2$]. Since in *iso*-lithobilianic acid ring opening has occurred between C_2 and C_3, such a finding would be in disagreement with the now accepted formulation. This difficulty has been satisfactorily cleared away by Langer (31), who found that lithobilianic acid, and not *iso*-lithobilianic acid, is formed from the purified dicarboxylic acid (XXIII) obtained from coprostanol. This



XXIII XXIV XXV

X = CO_2H

acid (XXIII) is stereoisomeric with the dihydro-Diels acid (XXV) (obtained from cholestane-4-one or by catalytic reduction of Diels acid from cholesterol and hypobromite), since the two acids furnish an identical ketone (XXIV).

Ergosterol

The experience gained in the elucidation of a complicated structure like that of cholesterol is naturally of great service in unraveling the structure of the closely related phytosterols and of ergosterol. Although a few years ago ergosterol was only a laboratory curiosity, the commercial interest aroused by its conversion into vitamin D has made it now available in large quantities. In spite of intensive studies, however, its constitution has not yet been elucidated with that degree of certainty which is essential for the formulation of the complex changes occurring in the production of vitamin D from ergosterol by the action of ultraviolet rays.

Since Tanret's discovery the formula $C_{27}H_{42}O$ had been attributed to ergosterol, the only sterol containing three double bonds. The introduction by Callow (32) of the 3,5-dinitrobenzoic acid esters for the separation of the irradiation products of ergosterol, which led to the isolation of crystalline vitamin D by Askew *et al.* (33), enabled also a re-examination by analytical methods of the molecular formula. The composition of ergosterol has now been established as $C_{28}H_{44}O$ (34) and analyses of bromo derivatives of certain derived ketones confirmed this formula (35).

The side-chain of ergosterol is now known to be

$$\cdot CH(CH_3) \cdot CH : CH \cdot CH(CH_3) \cdot CH(CH_3)_2$$

and to differ from that of cholesterol by a double bond and an additional methyl group. This fact was demonstrated by Guiteras *et al.* (36), who oxidised two reduction products of ergosterol, namely ergostenol and ergostanol, with chromic acid and isolated *l*-methyl *iso*hexyl ketone, $CH(CH_3)_2 \cdot CH(CH_3) \cdot CH_2 \cdot CH_2 \cdot CO \cdot CH_3$. The structure of this ketone follows in part from the results of oxidising ergosterol with ozone. In the hands of Reindel & Kipphan (37) *r-iso*propylmethylacetaldehyde, $CH(CH_3)_2 \cdot CH(CH_3) \cdot CHO$, was obtained, but Guiteras *et al.* (36) succeeded in isolating the optically active *laevo* form. Incidentally the position of the double bond in the side chain is hereby fixed.

The ring-system of ergosterol is the same as that of cholesterol since on oxidation of ergostane (the completely saturated hydrocarbon derived from ergosterol), an *allo*norcholanic acid was obtained, identical with the acid derived from cholesterol via *allo*cholanic acid by shortening of the side chain by one methylene group (38). This observation has been confirmed in a slightly different manner (39). Incidentally these results support the structure given to the side chain.

Hydroxyl group of ergosterol.—The allocation of this group to ring I follows from an observation of Reindel (40) that ergostanol (hexahydroergosterol) gives on oxidation a dicarboxylic acid, $C_{28}H_{48}O_4$, from which a pyroketone is obtained. The position of the hydroxyl group in ring I is uncertain; it is usually allocated to C_3 by analogy with cholesterol, but the observation of Heilbron, Samant & Simpson (39) that chloroergostane (hydroxyl group of ergostanol replaced by chlorine) on oxidation gives a chloro*allo*norcholanic acid different from the known 3-chloro*allo*norcholanic acid (prepared from

cholesterol) raises considerable doubt as to the correctness of this supposition.

Among the many interesting reactions shown by the labile molecule of ergosterol, one of the most remarkable is its conversion into a pinacone, $C_{56}H_{86}O_2$, by the action of light in the absence of oxygen, on its alcoholic solution containing eosin. On pyrolysis or by heating the pinacone in decalin solution, methane is evolved and a new sterol, called *neo*ergosterol, $C_{27}H_{40}O$, is formed (41). This substance differs from ergosterol by the elements of CH_4 and its properties indicate the presence of an aromatic ring. It was found that on oxidation with nitric acid it yielded benzene-1,2,3,4-tetracarboxylic acid, whereas ergosterol under parallel conditions gives toluenetetracarboxylic acid. In either case such an acid could only arise from rings II or III of the sterol structure and in the case of ergosterol this must have involved a migration of a methyl group, which is probably the one lost in the formation of *neo*ergosterol from the pinacone.

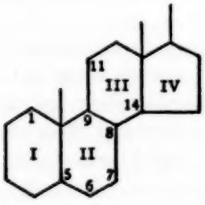
The ease with which the aromatic nucleus in *neo*ergosterol is formed suggests that in ergopinacone, and hence in ergosterol, the two nuclear double bonds are already situated in the ring which becomes aromatic, whilst the ultraviolet absorption spectrum together with the reactivity of ergosterol with maleic anhydride (42) demand a conjugated system of double bonds.

The constitution of ergosterol.—It proved quite impossible on the old formula of the sterols with a quaternary carbon atom at C_9, the meeting-point of three rings, to formulate the multiplicity of changes undergone by ergosterol. In the new cholane structure these changes are capable of interpretation in various ways and Rosenheim & King assigned the double bonds to ring II in the positions $C_5 : C_6 — C_7 : C_8$ (XXVI). On the other hand Windaus (43) preferred the positions $C_9 : C_{11} — C_8 : C_{14}$ in ring III (XXVI) for the conjugated system of double bonds, an arrangement also supported by Heilbron, Morrison & Simpson (44). The main arguments adduced by Windaus in favour of such a formulation are that, in the first place, it must contain a grouping $—CH : C<$ since the diol formed by addition of two hydroxyl groups to this double bond contains a secondary and a tertiary alcohol group, and, secondly, because the double bond of the maleic anhydride addition product of ergosterol apparently resisted catalytic reduction. On further examination of the latter point, however, it was found (45) that the double bond of the addition product is reducible and in consequence Windaus (46) rejected his previous

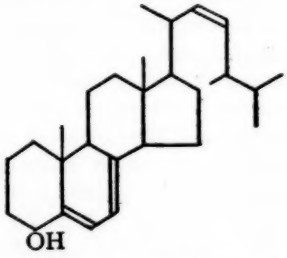
formula and now favours ring II for the conjugated system in the positions $C_6 : C_7 — C_8 : C_9$ (XXVI).

Such an arrangement, however, is open to the criticism that it does not contain the grouping —CH : C<, which is demanded by the reaction ergosterol → ergostadienetriol (see above). Moreover, it meets the requirements neither of X-ray analysis nor of the measurements of monomolecular films, and lastly offers no explanation for the intense ultraviolet absorption of ergosterol. Each of these physical measurements, typically characteristic for ergosterol, can be explained only by a formula in which the hydroxyl group is associated as closely as possible with a conjugated system of double bonds (47).

The available experimental evidence is definitely against the position of the OH group at C_3, whilst it does not exclude the one at C_4. The latter position in conjunction with the conjugated system of double bonds ($C_5 : C_6 — C_7 : C_8$) in ring II, as originally proposed by Rosenheim & King, fulfils the demands of the physical measurements and it seems therefore to the reviewers that the constitutional formula shown in (XXVII) offers the most satisfactory explanation of the known reactions of ergosterol (48).



XXVI



XXVII. Suggested formula for ergosterol

The irradiation products of ergosterol.—A bewildering amount of valuable experimental material has been gathered together during the last two years by Windaus and his collaborators in their study of the irradiation products of ergosterol. Nevertheless, mainly owing to the lack of a well-established constitutional formula of ergosterol, this work has hitherto not led to a clear understanding of the intramolecular changes underlying the conversion of ergosterol into the vitamin. That the process of isomerisation by ultraviolet rays occurs

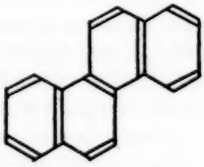
in at least three stages is evident from the isolation of five well-characterised isomerides of ergosterol, which are probably formed in the following order: ergosterol → lumisterol → tachysterol → calciferol → suprasterol I and suprasterol II. Of these crystalline substances only the product of the third stage, calciferol, possesses antirachitic activity, the last two being products of over-irradiation. The molecular formula proposed by Rosenheim & King makes it possible to formulate this series of isomerides, unique in organic chemistry, in such a way as to supply a much-needed working hypothesis (48).

Since of these substances ergosterol alone is precipitable by digitonin, it is probable that epimerisation of the hydroxyl group has already taken place at the lumisterol stage. In lumisterol, tachysterol, and calciferol a nuclear conjugated system of double bonds is still present, but how it differs from that in ergosterol is not known; in suprasterols I and II the conjugated system is absent. In all cases, however, the position of the double bond in the side chain is unaltered. This exocyclic double bond between C_{22} and C_{23} is not necessary for the production of antirachitic properties since 22-dihydroergosterol (46) can be activated by irradiation.

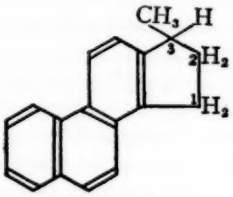
Aromatic hydrocarbons derived from the cholane structure.— Progress in sterol chemistry was delayed by the lack of a suitable method for breaking down the highly resistant molecule of cholesterol into smaller recognisable units, preferably aromatic hydrocarbons, which have so often yielded valuable information about the nature of complex ring systems. Mauthner's (49) first attack on the problem (pyrolysis of cholesteryl chloride) led to a mixture of hydrocarbons $C_{19}H_{28}$, evidently formed by the loss of the C_8H_{18} side-chain from $C_{27}H_{46}O$. But even when Diels applied his novel dehydrogenation methods to $C_{19}H_{28}$, cholesterol and cholic acid, no attention was paid to the successful isolation of chrysene. This obvious clue led five years later to the fundamental reconstruction of the old cholane formula. The patient methods of step-wise degradations and the neglect of the equally important evidence afforded by the more drastic method of Se-dehydrogenation had led to a plausible structure which not only failed to reconcile the results of the two methods of attack, but suffered also from the defects described on p. 91.

The formation of chrysene, $C_{18}H_{12}$ (XXVIII), from sterols and bile acids by the action of palladised charcoal, zinc dust, or selenium under fairly drastic conditions is now well established. Its production from the cholane nuclear structure is readily accounted for by ring-

enlargement of the five-membered ring IV at the expense of the tertiary methyl group at C_{13}.



XXVIII

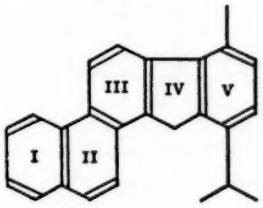


XXIX

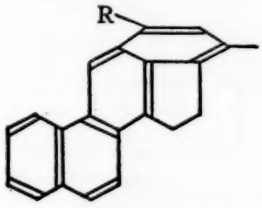
The formulation of the two other aromatic hydrocarbons, $C_{18}H_{16}$ and $C_{25}H_{22}$ (?), which were also obtained by Diels and his co-workers (6) from cholesterol and ergosterol by the dehydrogenating action of selenium, presents greater difficulties. $C_{18}H_{16}$ is clearly formed from cholesterol, $C_{27}H_{46}O$, by the loss of one CH_3 group and the side chain. It was shown by Rosenheim & King (3) that $C_{18}H_{16}$ was a phenanthrene derivative and they assigned the structure of a 3-methyl*cyclo*pentenophenanthrene (XXIX) (cf. 43) to the hydrocarbon, a formulation which implies a migration of a CH_3 group from C_{13} to C_{17} (numbers as in cholane formula). Other investigators considered the possibility of its being *cyclo*pentenophenanthrene, $C_{17}H_{14}$. The synthesis of the latter hydrocarbon by three different groups of workers (50) and Bernal's (51) comparison by optical and X-ray methods of the crystals of Diels' hydrocarbon with *cyclo*pentenophenanthrene proved conclusively that, although the two substances were closely allied in structure, they were not identical. The possibility of Diels' $C_{18}H_{16}$ being 1- or 2-methyl*cyclo*pentenophenanthrene was excluded by synthesis [Ruzicka *et al.* (50)]. There remains 3-methyl*cyclo*pentenophenanthrene, which has been synthesised by Bergmann & Hillemann (52). Although these authors found its properties to be closely similar to those of Diels' substance, they did not claim identity of the two hydrocarbons. Kon's (18) recent synthesis of 3-methyl*cyclo*pentenophenanthrene by an unequivocal method showed, however, that this hydrocarbon was indistinguishable chemically from Diels' original hydrocarbon, a conclusion supported by Bernal's X-ray crystal measurements.

The constitution of Diels' hydrocarbon, $C_{25}H_{22}$ ($C_{26}H_{24}$?), from cholesterol has not yet been definitely established. It is evidently a

pentacyclic hydrocarbon, and its properties agree with those of a fluorene derivative (formation of monoketone, etc.), as already recognised by Diels. Its formation is easily understood on the basis of the new cholane formula and Rosenheim & King (3) pointed out that ring formatoin can take place by linking up of the side chain with either C_{12} or C_{16}. Of these two possibilities they considered the latter



XXX



XXXI

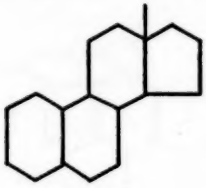
the more likely (XXX) since it accounts for the formation of a fluorene structure from ring IV. This formulation is also favoured by Cook & Hewett (50), who found, in confirmation of this view, that oxidation of the hydrocarbon with CrO_3 failed to yield an anthraquinone derivative. Such a derivative is obtained from methylcholanthrene (see p. 94) and should also be formed from $C_{25}H_{22}$ if ring closure with C_{12} had taken place.

Ruzicka and his co-workers (53) prefer formulation XXXI for the hydrocarbons from cholesterol and ergosterol ($R = C_4H_9$ and C_5H_{11} respectively), since their experimental results indicate that different hydrocarbons are obtained from cholesterol, ergosterol, and sitosterol by Se-dehydrogenation. In this connection it should be mentioned that the ring systems of cholesterol, ergosterol, and the two phytosterols, sitosterol $C_{29}H_{50}O$ (54) and stigmasterol $C_{29}H_{48}O$ (55), are identical (56). Apart from the distribution of double bonds in the nucleus, these sterols differ only in the number of carbon atoms in the side-chain, which also carries a double bond in ergosterol (37) and in stigmasterol (57).

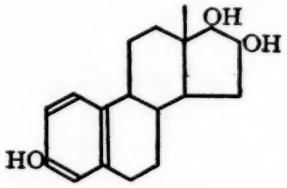
The relation of the sterols to oestrogenic hormones is clearly expressed in the formulation of the latter, based on the new cholane structure, and is emphasised by the discovery that ergosterol, calciferol, and especially *neo*ergosterol possess oestrogenic activity (58). Still more important is the newly found correlation between oestrogenic and carcinogenic properties (59), which raises the question

whether metabolic substances, originating from cholesterol or the bile acids, may not be concerned either directly or indirectly in the production of malignant tumours.

The oestrogenic hormones are derived from the parent hydrocarbon oestrane (XXXII) [for nomenclature see (60)] and evidence



XXXII



XXXIII

for the presence of a partially reduced phenanthrene nucleus in trihydroxyoestratriene (oestriol, theelol, trihydroxyoestrin) (XXXIII) has been forthcoming by its conversion into 1,2-dimethylphenanthrene (61), identical with the synthetic substance and with that obtained from aetiobilianic acid.

The introduction of the new cholane formula has led to a rapid solution of the constitutional problem and the way to synthesis now lies open. A beginning has already been made by the synthesis of *cyclo*pentenophenanthrene, the parent hydrocarbon of the cholane structure, and in other directions (62). The attack on the synthetic side of the problem is as promising of success now as it was sure of failure a few years ago, when even Wieland and Windaus admitted in their Nobel Lectures that the peculiar ring system of their formula defied attempts at synthesis.

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THE CHEMISTRY OF THE AMINO ACIDS AND THE PROTEINS*

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A comparatively recent epoch ascribed to the proteins the central position in the life processes. Today, however, they appear rather as the passive carriers of the life phenomena, determining the important chemical and physico-chemical properties of the medium and the mechanical properties of the tissue or forming the base for certain attached active groups. The true directive participation in the maze of vital chemical reactions on the other hand devolves on scattered specific substances which, in general, belong to entirely different classes of bodies and by which the fields of the chemist and biochemist have been greatly enlarged.

Just so much the more is it gratifying to realize that the proteins and their building stones engage the active interest of the experimenter. The direction which the experiments have taken has always been determined by the relation between the physico-chemical and the colloidal properties of the proteins and their chemical structure as has for years been predicted and represented (1). A more extensive monograph which recently appeared (*2a*) attempts to give as complete as possible a view of the physical chemistry of proteins. The strong influence which the modern electrolyte theory and especially the electrical constitution of the atom and molecule has had on experimentation in the field of the electrochemistry of colloids (*2b*), especially of proteins, is expressed in most new work on this subject.

Amino Acids

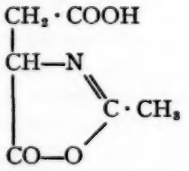
Let us first examine the contributions of the past year to the chemistry of the amino acids. In the realm of the synthesis of these acids, the carbobenzoxy experiments of Bergmann (3) and co-workers has been the greatest step forward. By means of these, Bergmann, Zervas & Salzmann (4) have now produced *l*-asparagine and *d*-glutamine. Carbobenzoxy-*l*-asparagine was changed into its anhydride. On heating with benzyl alcohol this yielded chiefly the α-benzyl ester and then, on treatment with PCl_5, the ester chloride. This was

* Received February 5, 1934.

changed into the ester amide with ammonia. During catalytic hydrogenation the carbobenzoxy group and the benzyl ester group were split off in one operation with the formation of the natural *l*-asparagine. The synthesis of *d*-glutamine was carried out in an analogous manner which makes this important substance readily accessible for further investigations. The authors propose to report later on the use of this method for the synthesis of α-peptides of glutamic acid to which glutathione belongs. As was mentioned in the previous review, they obtained by this method the *l*(+)-isoglutamine (3) with a rotation of $(\alpha)_D = +21°$. A great advantage of the method is that one obtains, without complications, optically pure amino-acid derivatives and peptides from optically active amino acids. Abderhalden & Nieburg (5) believed that they produced an *l*(+)-isoglutamine by another method, which, however, rotated in the opposite direction, $(\alpha)_D = 25°$, and assumed a Walden inversion in the synthesis of Bergmann & Zervas. The latter, however, were able to show (6) that Abderhalden & Nieburg did not have isoglutamine but had instead the ammonium salt of *l*(−)-pyrrolidone carboxylic acid.

A good method for the preparation of aspartic acid with a 59 per cent yield is described by Dunn & Fox (7), who start with diethyl-fumarate which gives a well-crystallized diketopiperazine-diacetamide with alcoholic ammonia at 100° and 6 atmospheres pressure, and which can then be easily hydrolyzed into aspartic acid by sodium hydroxide.

Harington & Overhoff (8) report a new synthesis of 2-thiolhistidine. Contrary to a structural formula of the product postulated by Bergmann (9) and his co-workers, on the basis of the effect of acetic acid anhydride on aspartic acid, Harington & Overhoff assume an azlactone of the accompanying formula. On treatment with PCl_5



with dry ether in alcoholic solution, there is produced an ethyl-α-acetamino-γ-keto-δ-chlorovalerate which is changed into the phthalamino derivative and which gives the chloride of α-δ-diaminodiketovaleric acid on heating with 20 per cent HCl. Treatment with an

equivalent of NaCSN gives the chloride of 2-thiolhistidine and then the free amino acid.

Richardson (10) carried out the electrometric titration of *dl*-2-thiolhistidine. This was possible with the hydrogen electrode using freshly platinized electrodes as well as with an indigo-carmine electrode but not with the quinhydrone electrode. He found the dissociation constants $pK_1 = 1.84$, $pK_2 = 8.47$, $pK_3 = 11.4$, corresponding to the groups COOH, $-NH_2$ and -SH. The differentiation between basic and acid constants was made after their displacement by the addition of acetone.

Wurmser & Mayer-Reich (11) have calculated the free energy of the synthesis of alanine from pyruvic acid and ammonia to be −16,500 cal.

The decomposition of amino acids has been widely studied. Wunderly (12) investigated the aminolysis of aspartic acid in a suspension of animal charcoal warmed to 80° C. It was estimated that it was about 61 per cent decomposed to NH_3, CO_2, lactic acid, malic acid, pyruvic acid, and acetaldehyde. The proportion of NH_3 to CO_2 which was equal at first was more and more displaced in favor of the NH_3. Kisch (13), who followed the oxidative deamination of amino acids by means of methyl glyoxal according to the method of Neuberg & Kobel with a special apparatus, found that alanine was more difficult to deaminize than serine and this more difficult than glycine. Very noteworthy observations on the autoxidation of amino-acid derivatives with animal charcoal and hemin were instigated by Bergel & Bolz (14). They found that leucine oxidizes with animal charcoal approximately ten times as rapidly as alanine. Acetylation of the amino group hinders the oxidation while the addition of an alkyl group hastens it. With N-methylalanine it proceeds 2–3 times as quickly as with alanine. N-dialkylamino acids (from glycine, alanine, and leucine) are easily oxidized completely with animal charcoal. The dialkylamino acids are not oxidized by means of hemin but the simple amino acids are. In kidney sections, on the other hand, the natural amino acids are not oxidized, while the dialkylamino acids are. The oxidation ferment of the kidneys, therefore, can have no relation to the hemin-like substances.

The action of formaldehyde on amines and amino acids was studied quite recently by Clarke, Gillespie & Weisshauss (15). Zeleny & Gortner (16) had shown that the heating of alanine, glutamic acid, cystine, and tyrosine with formaldehyde in 20 per cent HCl leads

to the splitting off of more than 40 per cent of the nitrogen as methylamine. The authors find a similar decomposition on heating amino acids with formaldehyde in the presence of formic and acetic acids. Under these conditions glycine yields 60 to 70 per cent of dimethylglycine together with considerable amounts of complex non-crystalline products and 6 per cent of volatile base (chiefly trimethylamine). β-Aminopropionic acid acts in a similar manner. On the other hand, alanine yields no dimethyl derivative; 36 per cent of the nitrogen is changed into methylamine. Leucine, α-amino caproic acid, and glutamic acid act in a similar manner.

A new method for the determination of histidine for use in the study of biological fluids has been devised by Kapeller-Adler (17). The black substance which appears on brominating histidine dissolves in concentrated ammonia with a purple-red color and in ammonium carbonate with an intensive blue-violet color. This color follows Beer's law and can be evaluated colorimetrically for quantitative determination by comparison with a standard solution. The reaction is specific and is sensitive to 1 : 50,000. Lang (18) described a micromethod for histidine which is founded on the capacity of histidine to form insoluble compounds with complex mercuric compounds in a dilute alkaline medium. It differs from tyrosine in this respect. The mercury mixture of Hinsberg & Laszlo (19) serves as the mercuric compound. The determination is made by the step photometer. The accuracy of this is quite satisfactory. Following the report by Abderhalden & Heyns (20) of the existence of *l*(+)-norleucine as a constituent of spinal cord and peripheral nerves, Czarnetzky & Schmidt (21) also obtained this substance. Abderhalden & Heyns proceeded with the method which had proved successful for *l*(+)-norvaline. Norleucine was not obtained unequivocally as a definite constituent of the brain protein.

Bauer & Burschkies (22) produced an insoluble compound I from cysteine and lead acetate which in the presence of Na_2CO_3 splits into $PbCO_3$ and a soluble lead-containing compound, which on acidification is converted to compound II, cysteine-lead. This is soluble in the presence of Na_2CO_3. As was shown by Klement (23) by various methods the lead in compound I is in a readily reactive ionic form and in compound II it is much more strongly bound because of the interposition of sulfur and complex binding with NH_2. Szendrö and co-workers (24) obtained *l*-cysteine with a yield of 5 per cent by the action of ultra-violet light on cystine in an atmos-

phere of nitrogen in acid and alkaline solution. The experiments were stimulated by a work of Wels (25), who found, by treating proteins with ultra-violet light in pure nitrogen, a substance which was identified by its characteristic property of acting as a reducing and oxidizing system. By means of careful new determinations of reduction potentials of sulfhydryl compounds (cysteine, glutathione, glycylcysteine) Green (26) attempted to take a stand in the controversy between Dixon & Quastel on the one hand and Michaelis on the other on the question of the mechanism of the oxidation of cysteine to cystine. He calls attention to the special position of this system which does not permit a simple conception of the reaction. The theory of Dixon makes allowance for this.

Saunié & Truhaut (27) tested the capacity of different amino acids to reduce mercury salts and found that glycine, serine, histidine, tryptophane, phenyl-β-alanine, dihydroxyphenylalanine, proline, creatine, creatinine, and cystine reduce the mercuric compounds, especially Nessler's reagent. This reduction fails in the case of alanine, valine, leucine, aspartic acid, arginine, phenylalanine, and glutamic acid.

In connection with the question of the irreversible change of gelatin on continued heating in aqueous solution, Gerngross & Deseke (28) studied the resistance of peptide compounds to thermolysis in aqueous media; among these were alanylglycylglycine, alanyldiglycylglycine, leucyldiglycylglycine, and leucyltetraglycylglycine. The increase of amino nitrogen with time (up to 9 days) was determined by the Van Slyke method. This hydrolytic splitting, which at first was unnoticeable and then became increasingly greater, was expressed as percentage of the total of such groups available. A higher molecular speed of hydrolysis was found with increasing length of the chain of the polypeptide. The slight initial degree of thermal hydrolysis of gelatin is ascribed to a certain protection afforded by the micellary nature of the aggregate which is later lost. Then the irreversible change takes place.

In an earlier work, Borsook & Huffman (29) gave an illuminating presentation of the method of calculating free energies of formation and appended the data for aqueous solutions of *d*-alanine, *l*-aspartic acid, and glutamic acid. They carried out further interesting experiments on the free-energy change of the reaction, *l*-aspartate$^{=}$ → fumarate$^{=}$ + NH_4^+, in which they compared the thermal data for this reaction with the equilibrium which they obtained by the action of micro-organisms. The values obtained agree quite satis-

factorily with each other. Nims & Smith (30) have determined the first and second ionization constants of *dl*-alanine in the range of 20° to 40° by means of electrometric measurements in a manner which is thermodynamically free from objections, and with great accuracy. From these data they calculated the heat of ionization. In the meantime there appeared the important article which was discussed last year: "Transference and Conductivity Studies on Solutions of Certain Proteins and Amino Acids with Special Reference to the Formation of Complex Ions between the Alkaline Earth Elements and Certain Proteins," by Miyamoto and Schmidt (31). It seems important that no foundation was found for the formation of complex ions between Ca, Ba, and Na with glutamic and aspartic acids. In the case of substances with so many and varied adherent groups on the molecule as the proteins, the transference number can give only a hint of the possibility of a complex bond of a cation but can give no definite proof of it. This can be determined only by other independent means. Data have been obtained, for example, for compounds of copper, iron, and silver, etc. Several interesting observations on the electrical charge on microscopic crystals of amino acids were made by Abramson (32).

Wyman & McMeekin (33) make a worthwhile contribution to the knowledge which we have up to the present time concerning the dielectric constants of solutions of amino acids and peptides. The former also examined several β-, γ-, and ε-forms, while the latter investigated the series of glycine peptids up to the hexapeptide. A linear increase of the molecular dielectric constant, δ, with increasing distance of the opposed groups was in agreement with the zwitterion theory. Furthermore, only a slight decrease of δ was observed for glycine in an alcohol-water mixture (to 60 per cent alcohol), which indicates a large preponderance of zwitterions in the mixture. A subsequent paper by these authors (34) shows that the dipole moments of the ethyl esters of a number of amino acids in benzene solution gave small values, which are not in proportion to the lengths of the chains. This disappearance of the relations noticed with amino acids suggests, on the other hand, the zwitterion theory, since the chain length and structure lose their meaning immediately on the exclusion of formation of zwitterions. Frankenthal (35) gives an amplification of his earlier work on the dielectric constants of aqueous solutions of several amino acids and polypeptides.

Edsall & Blanchard (36) calculated the proportion, K_z, of "zwit-

terions" to "uncharged molecules" from the dissociation constants of the amino acids and their esters by means of the fundamental method of Ebert (37). They reported titrimetric determinations of the constants for 8 amino-acid esters and for ε-amino propionic acid. The high K_s values ($4.10^{-5} - 1.10^{-6}$) show that the form $A^{\pm}$ preponderates in aqueous solution. With an increase of temperature K_s rapidly decreases. With decreasing dielectric constant of the medium, the concentration of the zwitterions decreases and that of the uncharged molecules becomes greater than in water, although in 90 per cent alcohol the $A^{\pm}$ form is still significant. The results of this beautiful work are also of interest for the theory of the heat coagulation and alcohol precipitation of proteins. An explanation has been sought for some time (38) from the change of the ionogen into the neutral and finally into the anhydride-like form on the basis of the work of Bjerrum and Ebert.

Failey (39) determined the change in the solubilities of $TlIO_3$ and TlCl by means of several α-, β-, and certain nitrogen-substituted acids, as well as of glycylglycine and urea. If S/So represents the ratio of the solubility of the salt in the amino-acid solution and in water, then we have a linear function of log S/So when plotted against the concentration C of the amino acid instead of $\sqrt{C}$ as in the case of the strong electrolytes. The differences in behavior of the various amino acids are not determined by the dielectric constants.

Proteins

Pigments.—Among the proteins, the blood pigments have been widely and successfully studied. Roche (40, 41) differentiates between two distinct kinds of pigments of the blood-pigment type: (*a*) Blood and muscle hemoglobin and (*b*) the chlorocruorin of various polychaete worms. In the hemoglobins the differences are dependent on the globin. The protohematins are the same. Protohematin from any source gives with any definite globin the same spectrum; the amounts of oxygen and carbon monoxide which it can bind also equal those of the hemoglobin which was used for the preparation of the globin. Muscle hemoglobin acts in the same way since in the coupling of the protohematin only the globin is determinative for the specificity of the hemoglobin obtained by this coupling. Determinations in O_2-CO mixtures showed that muscle hemoglobin possesses a much slighter affinity for CO than blood hemoglobin. With helicorubin, with the intracellular coloring matter of yeast,

various mussels, crabs, and actinia, as well as with cytochrome, we are dealing with an analogous combination of protohematin and globin-like proteins. With chlorocruorin, on the other hand, the differences depend on both components. Crystallized hemoerythrin, which is easily denatured by strongly alkaline or acid reactions (42), resembles the globulins and has an isoelectric point of 5.85. The electrotitration curve resembles that of the hemocyanin of *Helix*. Svedberg & Ericson (43) propose the name erythrocruorin for the erythrocyte protein of the invertebrates; by means of the ultracentrifuge they determined the pH range of stability and the molecular weight of the erythrocruorin of *Arenicola marina* (I) and *Lumbricus ferrestris* (II). The former possessed a molecular weight of 2,850,000 and the latter 2,730,000. The isoelectric points were 4.56 (I) and 5.3 (II). The great differences between these values and those of hemoglobin (M.W. 68,000; isoelectric point, 6.7) are obvious. Sørensen & Sørensen (44) examined thoroughly the solubility and tendency toward dissociation of CO-hemoglobin (horse) in ammonium-sulfate solution. They took into consideration the water content of the crystallized hemoglobin which had been purified with the greatest care. The influence of the $(NH_4)_2SO_4$ concentration on the equilibrium between the crystallized hemoglobin and the mother liquor led to a satisfactory confirmation of the Cohn equation for the solubility in salt solutions, as in the analogous researches of Green (45). Besides a minimum solubility in the isoelectric range, pH = 6.6, there is a second point dependent on the pH, viz. at pH = 5.4, which corresponds to the formation of a difficultly soluble sulfate. It may be concluded from the influence of the hemoglobin concentration on the equilibrium between the mother liquor and the crystalline hemoglobin, as well as from the impossibility of fractionation, that hemoglobin, like egg albumin and unlike the serum proteins, shows only a slight dissociation tendency (46). Apropos of this important finding, it does not seem superfluous to stress repeatedly, in accordance with Sørensen, that these facts are valid only under defined conditions. They cannot be applied to highly purified, electrolyte-free proteins or to proteins in the native state without some modification since the same pH with or without the presence of salt can mean something entirely different relative to the condition of the protein.

Although the respiratory blood pigments of the lower animals of the same class constitute well-defined substances of almost uniform molecular weight according to the ultracentrifuge experiments of

Svedberg and co-workers (43, 47), nevertheless, the careful electrophoretic researches of Pedersen (48) show that their isoelectric points lie definitely apart, which points to definite differences in amino-acid distribution.

Casein.—Cherbuliez & Schneider (49) have added a new attempt to efforts previously made to fractionate casein. They shook 5 gm. of casein in 500 cc. of 5 per cent NH_4Cl at a pH of 4.6, whereupon a soluble and an insoluble fraction were separated. From the soluble fraction they obtained by repeated precipitation with acetone as well as by extraction with acetone and ether in the Soxhlet apparatus, a white powder, β-casein. α-Casein was obtained from the first NH_4Cl-insoluble fraction by purification. Natural casein, α- and β-casein, when jointly dissolved in 0.01 NaOH, have $[\alpha]_D = 111°$. α- and β-casein show the same equivalent weight in titration with phenolphthalein, but exhibit otherwise great differences with respect to salt solution as well as with respect to solubility in 70 per cent pyridine. Furthermore, β-casein is only slightly coagulated by rennin. In further researches carried out with Meyer (50) the fractionation was carried out further to four chemically characteristic entities, viz. casein α_I, α_{II}, β, and γ, which, they claimed, were not split products but primary components contained in varying amounts in different caseins. In this connection it might be worth mentioning that Demanez (51) was unable by fractionation of cow's milk casein by means of hot acidified alcohol (using the method of Linderström-Lang) to show any difference in the precipitin reaction with antiserum. By means of sheep-casein antiserum, sheep, cows', and goats' milk could be differentiated by their positive precipitin reactions from human and mares' milk.

By continued heating of casein with 2.5 *N* HCl and further hydrolysis, Lipmann (52) was finally successful in isolating serine phosphoric acid, previously obtained from vitelline (53), as a tribasic Ba and Ag salt. The velocity constants for the hydrolysis of phosphoproteins and serine phosphoric acid agree, which strongly points to the idea that serine forms the only group in these proteins which unites with phosphoric acid.

Fibrinogen.—In a report on fibrinogen and a plasma globulin which promotes coagulation, Schmitz (54) shows that the classic method of preparing fibrinogen according to Hammarsten (half saturation with NaCl) and the method of Mellanby (dilution of the plasma with distilled H_2O and weak acidification with acetic acid)

produce entirely different products. The Hammarsten fibrinogen cannot be fractionated by salting out. The Mellanby fibrinogen can be separated by half saturation with $(NH_4)_2SO_4$ into an apparently unitary fibrinogen and a precipitable globulin which promotes clotting. The latter would seem to be identical with Mellanby's prothrombase.

Fibrin.—Katz & de Roy (55) have determined by X-rays the spacings of the lattice planes of fibrin which contain crystalline micelles and show a certain similarity with the X-ray spectrum of keratin.

Gelatin.—Fodor & Kuk (56) continued their cleavage studies with gelatin heated in glycerol at 130° and obtained complexes of hexa- and dipeptides.

The formation of a phenol-protein compound, assumed by Spiro (1920), is probably established by the work of Sarluy (57) by means of viscosity measurements on gelatin in varying phenol-water mixtures at 70°, which gave a marked increase in viscosity. This was probably due to the formation of a phenol-solvation shell around the micelle.

Malone (58) has made satisfactory pH measurements in 0.5 per cent and 1 per cent gelatin solutions by means of the glass electrode, although to be sure there have been no difficulties in making such measurements with other electrodes.

Custers, de Boer & Dippel (59) bring us noteworthy observations on the absorption spectrum and the dispersion of light in isoelectric sols and gels of gelatin. These indicate a stable sol-form, A, at higher temperatures and a stable gel-form, B, at lower temperatures. For gelatin sols in the pH range 2.5 to 8.6 there is a uniform absorption, while the dispersion reaches a maximum at the isoelectric point. Over a considerable range of pH the variation in the dissociation of the COOH and NH_2 groups is considerable, from which one may infer that these groups (in the range measured to 2481 Å) do not influence the absorption, while the optical rotation changes markedly with the pH.

Katz & Wienhoven (60) made a comparative study of the effect of various lyotropic substances on mutarotation and gel formation in gelatin-water mixtures. As a result of researches on the dispersion of light Duclaux & Hirata (61) assume two forms in gelatin sols and gels, one with a strong dispersion of white light and one with a weaker dispersion of blue, which are dependent on the pH in different ways. Krishnamurti (62) found that the Tyndall effect with the swelling of 20 per cent gelatin first increases and then decreases.

He ascribes this to absorption of the water, first by the micelles, later by the intermicellary spaces.

Jermolenko & Lobanowitsch (63) found that hydrotropic substances which aid swelling (KCNS, KI, and urea) on the one hand, and alcohol, phenol, and formalin on the other, are mutually antagonistic. Of the latter, formalin is the most active.

Collagen.—Jordan-Lloyd, Marriot & Pleass (64) compare the swelling of collagen fibers and 33 per cent gelatin. The general processes in the acid and alkaline ranges remote from the isoelectric point are similar and are hindered by salts. Collagen fibers swell less than gelatin and do not change their volume in a wide range around the isoelectric point. Collagen fibers consist of gelatin molecules, which are laid down parallel to the longitudinal axis and are held together by the strength of their lateral valences. Katz & Weidinger (65) found relationships analogous to those of starch with respect to the influence of salts and organic compounds on the contraction of collagen. The results of X-ray experiments on gelatin have recently been discussed by Hess & Trogus (66).

Edestin.—Florence & Pozzi (67) give a preparation of purified edestin which was obtained from hemp seeds by extraction with 5 per cent NaCl at 60°. The high total nitrogen content of 18.7 per cent and the decrease of ultra-violet-light absorption by weak hydrochloric-acid solutions were regarded as criteria for purity.

Gliadin.—Sinclair & Gortner (68) studied the peptization of gliadin by solutions of inorganic salts. On the basis of the chemical differences which lie within the limits of error and the constancy of free amino and carboxyl groups they come to the conclusion that the products which differ in their solubilities are not the result of a chemical heterogeneity of the protein molecules but of physical differences in the protein micelles. The solution by salts, which follows the anion series $I > Br > Cl$, does not depend in this on a true hydrolysis of the protein.

Carpenter & Lovelace (69) determined the isoelectric point of the crystallized globulin from orange seeds as $pH = 5.23$ by means of the electrophoretic method.

Keratin.—In an original manner Speakman & Hirst (70) have used the effect of pH on the work expenditure necessary to bring about a 30 per cent expansion of wool fibers to determine the range of stability of the keratin molecule. It lies approximately between pH 4 and pH 8. In the consideration of the connection between

swelling and the pH and its depression by salts, the dependence on the activity of the protein salt does not seem to have been considered. The authors come to the conclusion that "salt linkages" between the adjacent ionized amino and carboxyl groups play an important rôle in the structure of the keratin molecule. That the zwitterion structure can lead to strong lattice forces in dry protein has been discussed repeatedly since the first mention of it in the classic work of Bjerrum. In aqueous solutions one can expect those interionic forces in zwitterion structures to undergo corresponding modifications as in the case of other dissolved electrolytes. It might perhaps be confusing to speak here of salt linkages. Indeed only one hydrolysis constant can be derived from the dissociation constant. The strongly schematic considerations of the article have called forth a lively discussion (70).

Astbury (71) has worked on the X-ray analysis of animal hairs and other protein fibers. He develops a structural representation for the main and side chains of silk fibroin, on the one hand, and of wool and hair keratin on the other, and of feather keratin, which lies between the two groups, on the basis of the identity periods. After further researches with Marwick, a period of 309 Å was determined for feather keratin, corresponding to a length of 100 amino-acid residues. Such lengths make it possible to understand the appearance of the reversible mechanical effects in the protein fibers. To these observations, which are comparatively the same for gelatin, collagen, and muscle fibers, may be ascribed the flexibility and capacity for rotation of the main and side chains as first postulated by Meyer.

Trogus & Hess (72) report on X-ray studies on natural silk and its behavior with acids and bases. Ohara (73) brings us some very instructive illustrations of his microscopic observations on the swelling of silk fibroin in concentrated neutral-salt solutions (Weimarn), mostly in Ba $(SCN)_2$.

Plastein.—Folley (74) obtained purified plastein from Witte's peptone by treating with pepsin and repeatedly dissolving and precipitating it. On shaking with an excess of α-naphthylisocyanate, he was able to separate it into two distinct fractions. By means of alcohol extraction it was possible to obtain two alcohol-soluble fractions, which could be separated into three sub fractions.

At this point it is possible to mention only briefly the communications on the characteristics of the proteins of soya beans by Jinuma & Mashino (75) as well as those on the protamines of several kinds of fish by Lissitzin & Alexandrowskaja (76).

By the use of the step photometer, and above all by the use of time curves to characterize the reaction velocities of the various carbohydrates, M. Sørensen & Haugaard (77) have worked out the orcin method (78) as a quantitative procedure for the determination of the kind and number of the carbohydrate groups contained in the various proteins. The conclusions are as follows: a repeatedly recrystallized egg albumin contained 1.71 per cent mannose; an easily soluble serum-albumin fraction, recrystallized several times, contained 0.74 per cent of a carbohydrate consisting of equal parts of mannose and galactose; a difficultly soluble fraction contained in all only 0.02 per cent of carbohydrate; a horse-serum globulin which had been precipitated several times contained 1.82 per cent of carbohydrate (equal parts of mannose and galactose); well-purified casein contained 0.31 per cent of galactose, lactalbumin 0.47 per cent of galactose (in both of these there was no lactose); a difficultly soluble, repeatedly precipitated wheat gliadin contained 0.2 per cent of mannose. Lieben & Bauminger (79) studied the oxidation of casein and serum albumin by potassium permanganate and gave a synopsis of the nitrogen distribution with reference to melanin-N, ammonia-N, basic-N, and monoamino-N. An appended article is concerned with the oxidation of amino acids and proteins by sodium hypochlorite.

Finally, papers predominantly devoted to the more general protein problems should be discussed. Jordan-Lloyd & Phillips (80) thus summarize their views on the hydration and structure of proteins:

1. The hydration of proteins in water is due to the co-ordination of water molecules with the oxygen, nitrogen and hydrogen atoms of the hydroxyl, carboxyl, amino, amido, and, to a lesser extent, the imino and keto groups. Water-soluble proteins contain high proportions of the residues of serine or other hydroxy acids, lysine, arginine, and proline.

2. At the isoelectric point, protein molecules carry an equal positive and negative charge. Salt formation, between adjacent protein molecules, and between oppositely charged groups of the same molecule, will not lead to hydration owing to the equal size and valency of the two ions involved.

3. In acids and alkalies, the two ions of the protein salt produced differ enormously in valency, size, and the readiness with which they hydrate. This leads to increased hydration by the orientation of water molecules around the charged centers in the protein molecule. Acid-soluble proteins contain the residues of lysine, arginine, and, to a less extent, histidine. Alkali-soluble proteins contain the residues of aspartic and glutamic acids and, to a less extent, the amides of these acids.

4. The hydration of protein molecules in solutions of neutral salts is promoted because the protein molecules become associated with the small ions of the salt.

5. If protein ions exist in a system under restraint while other ions are freely diffusible, a Donnan equilibrium will also affect the distribution of the water.

6. Solubility and hydration of proteins is greatly affected by the closeness of packing of the molecules into organized structures with cross linkages between the molecules. Cross linkages involving co-valent bonds will be more resistant than those involving electro-valent bonds. Fibrous proteins, particularly those with a preponderance of residues of the short-chain aliphatic amino acids, are the most resistant to solvents.

7. With the development of oriented structure in proteins, a stability range toward hydrating influences appears. This range extends from about pH 4 to 8.

8. The pH value at maximum hydration in acid solution moves continually to a lower figure as the protein structure becomes more and more fibrous in character. The pH of maximum osmotic hydration in alkaline solutions becomes greater under the same conditions.

We should add to these remarks that unfortunately the purely hypothetical character of some assertions is not stressed sufficiently. For example, it is more than doubtful that just the H-, N-, and O-atoms of the hydrated groups are capable of adding on water molecules. The hydration influence of neutral salts is dependent on the concentration but the concept of an increase in hydration as a consequence of the association of the small salt ions is not the only possible mechanism; in this form it seems hardly probable to the commentator. That the difference between hydration and swelling is not kept sufficiently clear is emphasized in the discussion by Katz. We may here mention a later treatment (*2a*) of the hydration of proteins.

Greenberg & Greenberg (81) have investigated the question of hydration (bound water) of biocolloids by an ultrafiltration method to which solutions of gelatin, casein, starch, glycogen, and blood serum were subjected. According to them only very small amounts of water were bound by these colloids. As reference substances in the attempts, urea and sucrose were used, from the concentrations of which the "bound water" was estimated. Opposed to the method of these authors, Bull (82) employs in the calculation of the results a formula which takes into consideration the absorption of the reference substance by the colloids under examination; a very small degree of absorption could cause large errors. In any case further and more exhaustive experiments are necessary.

In connection with these studies on proteins an article by Gross (83) on the salting-out of non-electrolytes from aqueous solutions is of interest. Attention is called here to the additivity of the individual ion effects and to certain anomalies which are understandable

if one assumes an antagonistic ion effect, whereby probably the cation is responsible for the "salting-out" part and the anion for the "salting-in" part of the effect.

An entirely identical theory with the same co-ordination (*Zuordnung*) of an additive and an antagonistic ion-effect for the neutral-salt relations of proteins had been presented in 1902 (84).

Nasch (85) attempted to show for the inhibitory action of alkali in alcoholic precipitation that the amount of alkali which hinders the precipitation corresponds to the free amino group in the terminal position (crystallized egg albumin; albumin and globulin from horse serum).

Several years previously (86) the assumption was made that in the electrochemical building up of highly purified protein solutions with a hydrogen-ion activity lying on the acid side, the hydrogen ions are to be regarded as belonging in part to the excess A^- groups of the protein, and in part to be scattered statically over the protein molecule so that besides the polyvalent zwitterions with a negative excess charge, A^-, there will also be formed ions with positive excess charges, A^+. In fact, in considering the hydrogen-ion activity and the related conductivity, one finds that there is a plus conductivity which corresponds to these A^+ and A^- parts. Exact experimental data are now given (87) for highly purified solutions of ovalbumin, serum albumin, and pseudoglobulin, together with further data on gelatoses (88, 89) which show that the effect decreases from the more strongly acid ovalbumin to serum albumin, as is to be expected, and is lacking in the case of pseudoglobulin. Furthermore, a CO_2-reaction of the protein is not to be considered as the cause. It was further discovered that the fraction $^*A^-$ (without excess charge) of the whole protein becomes constant with higher protein concentrations, as is to be expected from theoretical considerations. In the case of the strongly acid, highly purified gelatoses the expected relation of hydrogen-ion activity and conductivity is also found. Errera & Hirshberg (90) determined potentiometrically by means of glass and antimony electrodes the change in pH during alkali- and acid-titrations of egg albumin, serum albumin, hemoglobin, and caseinogen, which had been purified by electrodialysis. The graphic presentation of Errera (91) is new, in that against the pH as abscissa the Δ pH, in millivolts, of two consecutive points are plotted as ordinates. This procedure permits us to recognize definitely the position of the isoelectric point. However, the maxima so obtained do not correspond,

as the author believes, to the maximum reactivities of the NH_2- and COOH-groups of the proteins.

The important series of experiments on electrokinetic phenomena conducted by Abramson and co-workers were carried out by the method described by Daniel (92) in which measurements of the speed of migration of quartz particles covered with egg albumin, gelatin, and gliadin in alcohol-water mixtures were made. The values were found to be in linear dependence on the strength of the field and independent of the form and size of the particles. They show only the relations of the protein film. The speed, $v_m\eta/\eta_0$, corrected for η, showed itself to be proportional to the bound H^+ of the gelatin in 35 per cent as well as in 60 per cent alcohol for the particles covered with gelatin. From this it was concluded that the gelatin, oriented with the active groups toward the liquid, becomes adsorbed on the quartz. In the presence of ethyl alcohol the isoelectric point appears to be shifted toward increasing pH in almost linear fashion with the dielectric constant, as is likewise the case with certain amino acids under similar conditions. In the opinion of the reviewer one should be able to observe the influence of the opposed ionic activities on the free charge, with a moderate shift in the position of the isoelectric point, in alcoholic solutions much sooner than in pure water. The very distinct decrease in the corrected v-values with increasing alcohol content (Table I) points definitely in this direction.

From very exact freezing-point determinations of solutions of crystallized ovalbumin and serum albumin on the addition of NaOH, NaCl, and $NaHCO_3$, Hedelius & Reinius (93) showed that the activities were decreased by the protein. With increasing pH values from 6.5 to 8.6 the activity coefficient of Na^+ in sodium albuminate solution decreases from about 0.9 to 0.6. The *fa* of NaCl also experiences a slight decrease between pH 5 and 7, which indicates a simultaneous Cl inactivation. Solutions of protein containing $NaHCO_3$ show relations similar to those of the corresponding sodium albuminate solutions.

Hughes & Rideal (94) had given a method whereby very small weighed amounts of proteins were spread out on a surface and the film of gliadin so formed was elegantly investigated by the determination of surface pressures and surface potentials. Hughes (95) now gives us the results of further investigations of this kind on the protein films of glutenin and ovalbumin, whereby the pressures and potentials of the films are expressed in terms of γ (gm. per sq.

cm.). The point of the (two-dimensional) sol-gel change for all three proteins is determined as about 1.4×10^{-5} gm. Several comparative observations on tridimensional gel formation by gelatin are also given.

Fosbinder & Lessig (96) are at present applying the same method to ovalbumin, serum albumin, and edestin and have determined the dependence of the surface potential on the surface concentration. They adhere, like Schulman & Rideal (97) and Matsui (98), to the double-form theory of protein structure as set up by Astbury & Woods (99): on the one hand, stretched-out polypeptide chains zig-zag in type (β-keratin form) and, on the other hand, the diketopiperazine-ring type (α-keratin form) which forms the more thickly packed films and leads to denaturation with a loss of solubility. In the work of Schulman & Rideal, which deals with the process of hydrolysis of protein films and the formation of monolayers of the proteolytic ferments themselves, we find a detailed review of the pertinent questions. Loughlin, using the "maximum bubble-pressure" method, found that solutions of 0.3 per cent ovalbumin and of 0.2 per cent and 2 per cent hemoglobin experience decreases in surface tension by careful heat denaturation which are not independent of the pH. This confirms earlier experiments. In a previous work (101) he found that careful heat denaturation does not change the titration curve of egg albumin, which, as the reviewer might remark here, in no way argues against the view that heat coagulation leads to an anhydride-like reaction of the side (ionic) groups of the protein molecule. Loughlin's results are of immediate concern to the question of the reversibility of this process. Similar relations are to be expected in the above-mentioned surface denaturation. The primary change (or at least so it seems as carried out repeatedly by the reviewer), which is the first step in any denaturation, would be the increase of the zwitterion structure. In a short communication by Cohn and co-workers (102), which concerns itself with very notable researches on the dependence between the zwitterion structure and molal volume of the amino acids, there is given a conception of the denaturation change which in part touches on the foregoing. One may look forward to the detailed presentation with the greatest of interest.

The speed of denaturation of crystallized egg albumin preparations of varying degrees of dryness was determined by Barker (103) and, in agreement with earlier works, was found to decrease

with decreasing water content. The temperature of denaturation was taken as that temperature at which (for a given period of heating) half of the protein became water-insoluble. It was found to be a linear function of the relative amount of moisture with which the protein was in equilibrium. Denatured protein takes up about 80 per cent of the amount of water which would be taken up by undenatured protein at the same relative humidity.

In a short communication Flosdorf & Chambers (104) report the surprising chemical actions of "audible sound (frequencies, 1000 to 15,000)," until now unknown; among these they mention the almost instant coagulation of Merck's egg albumin and of egg albumin recrystallized four times, as well as of plastein at 30°. More results are to be expected. Ettisch & Schulz (105) have been occupied with researches on the alteration with time in the uptake or loss of H^+ by proteins and the cause thereof. Like a number of earlier authors they base their experiments, which first were carried out potentiometrically with serum albumin and globulin of cattle, on the zwitterion theory, first formulated by Bjerrum for amino acids. For the effect of alkalies they found that there was an increase with time in the amount bound, with accompanying frictional and pH changes, in the case of casein and serum albumin, a phenomenon which has been known for some time. These authors separate the relation in a convenient way into two processes—a primary one taking place instantly and a secondary one extending over hours. For the application of the law of mass action and the calculation of the dissociation constant of native proteins, only the primary reaction can be used. To account for alkali- and acid-binding, three basic and two acid groups were assumed for 2 per cent albumin. To each of the groups was assigned a dissociation constant, derived on the basis of the Bjerrum equation, as well as a determined normality. With the ten values so derived, it was possible to reproduce the titration curves. The five reactive groups probably belong to definite amino acids. One might overlook here the observation that analogous ways of determining various dissociation constants of a protein were previously first described by Cohn and Simms, and the importance of diamino- or dicarboxylic-acid groups was stressed. Much has been said against the reality and assignment of such assumed dissociation constants (106, 2*a*), which is not disproved by Schulz & Ettisch. From the hypothesis of these authors it is conceivable that at extreme (high and low) pH values, therefore in the range of the combining

maxima, the zwitterion-reaction mechanism of either adding on or giving off hydrogen ions is important. In a special article (107) they describe the increase of acid combination with time in the range 21° to 37° as a consequence of a secondary reaction of ovalbumin.

Przylecki (108) with his co-workers has carried out further his researches on binding by the biocolloids and has gathered a large amount of material on protein compounds, chiefly by means of precipitation reactions. His work deals with compounds between proteins and nuclein and their decomposition products, with synthetic nucleoproteids, with compounds of proteins and polysaccharides, phosphoric acid, guanidine, and creatine, and they are attempting gradually to arrive at general conceptions. Büttner (109) reports on the combination of organic bases with proteins. The uptake of histamine and adrenalin chloride by gelatin and casein was studied by means of ultrafiltration and its dependence on pH was determined. The peptisation of casein by means of adrenaline was also examined. It was found that there was a constant relation between adrenaline and casein which was independent of the substrate in solution. Mutzenbecher (110), by use of the ultracentrifuge, is engaged in Svedberg's laboratory on the important question of the association and splitting of proteins. The studies seek to determine the difference, from the speed of sedimentation, between the proteins in untreated serum, in diluted serum, as well as after salt-fractionation, separation, and purification. These experiments are full of promise. For example, it was possible to obtain two fractions from globulin which differed greatly in their solubility but not at all in their speed of sedimentation, while a widely distributed homogeneous albumin showed itself as strongly polydisperse. Salt action leads partly to splitting and partly to aggregation; even dilution causes a change in the serum proteins.

In this connection mention should be made of the more recent researches of Bendien & Snapper (111), who prepared ultrafiltrates of serum of varying protein content by means of ultrafilters of differing permeability and determined the relation of albumin to globulin in these filtrates. Their results contradict the idea of an albumin-globulin complex, which, for some time, has been also denied by Adair & Robinson on the basis of osmotic pressure determinations. The calcium content of the ultrafiltrate ran parallel to the albumin fraction.

In icterus, bile pigments and albumin run parallel. Lecithin and

cholesterol seem to be connected in part with the euglobulin fraction, as are probably also the carriers of the Wassermann or Meinecke reaction in lues-serum.

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THE CHEMISTRY AND METABOLISM OF THE COMPOUNDS OF PHOSPHORUS*

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It is not possible in the short space available to deal with more than a relatively small number of the many interesting investigations which have been published during the last two years in this constantly expanding field. The reviewer has therefore followed the policy adopted in the previous review of limiting his ambit to a few only of the more active sections.

Recent Reviews

Amongst reviews and monographs, the following may be mentioned: Robison's (1) Herter Lectures on the significance of phosphoric esters in metabolism; Theiler & Green's (2) review of aphosphorosis in ruminants; Kay's (3) review of phosphatase in growth and disease of bone; the reviews of Barr (4) and Meyer zu Hörste (5) on pathological calcification (i.e., usually phosphorification also).

New phosphorus compounds.—The following have been isolated from natural sources: phosphoglyceric acid by the action of dried yeast on a mixture of glucose, hexosediphosphate, and acetaldehyde, in presence of sodium fluoride [Nilsson (6)] and by the action of a macerated muscle preparation on hexose phosphate [Embden, Deuticke & Kraft (7)]; mannose monophosphate from the mixture of phosphoric esters produced during the fermentation of glucose by yeast juice [Robison (8)]; fructose-1-phosphate from the partial hydrolysis of fructose diphosphate by bone phosphatase [Macleod & Robison (9)]; phosphoserine or serinephosphoric acid from casein and from vitellin [Lipmann & Levene (10); Lipmann (11)].

The Phosphoproteins

Although phosphorus is probably not a normal constituent element of all proteins, its presence in many carefully purified proteins is, of course, well established, even after all possible care has been taken to remove traces of lipin. The existence of one or more

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phosphorus-containing amino acids or other relatively simple phosphorus-containing building stones in the molecule of such proteins is an obvious possibility. In the case of casein and vitellin of egg-yolk, where the phosphorus is present to the extent of from 0.7 to over 1 per cent, several attempts have been made from time to time to isolate the phosphorus-containing fraction of the molecule with as little liberation of free phosphate as possible. Up till recently these have only succeeded to the extent of the isolation of soluble or insoluble protein-hydrolysis products containing a much larger proportion of phosphorus than the original phosphoprotein but still of relatively complex constitution, such as the vitellinic acid (from vitellin) of Levene & Alsberg (12), the phosphopeptone (from casein) of Rimington & Kay (13) (which was later found to contain hydroxyglutamic acid, hydroxyaminobutyric acid (?), and serine [Rimington (14)], the phosphopolypeptide prepared from casein by Posternak (15), and similar compounds from vitellin and from the ichthulin of pike spawn by Posternak & Posternak (16, 17). All of these polypeptides have been found to contain relatively large quantities of serine. The phosphopeptone was readily hydrolysed by phosphoric esterases (13) and it thus became a matter of the highest probability that the phosphorus of the phosphoproteins (or at least of their partial-hydrolysis products) was combined with the hydroxy group of one or more hydroxyamino acids in the form of an ester of orthophosphoric acid.

Lipmann has recently described in a short series of papers [Lipmann & Levene (18); Lipmann (19, 20)] the isolation of a simple ester of this type, serinephosphoric acid $NH_2 \cdot CH(COOH)$ $CH_2O \cdot PO \cdot (OH)_2$, both from casein and from vitellin, by partial hydrolysis of the phosphoproteins (or their derivatives) with dilute HCl, to which the serinephosphoric acid is relatively stable. There is little reasonable doubt that this substance is one of the genuine constituent amino acids of both of these phosphoproteins, whatever the linkage or linkages may be which normally unite it to the rest of the protein molecule. The serinephosphoric acid so prepared appears to be optically active. Like casein or vitellin it is readily dephosphorised by dilute alkali, and is fairly stable to acid.

Serinephosphoric acid (perhaps a more convenient name would be phosphoserine) is the first phosphorised amino acid to be isolated from natural sources. From the yields obtained by Lipmann it appears by no means impossible that others might exist in the protein

molecule. Lipmann states, however, that this is unlikely, and from the consideration of the velocity constants of the rate of liberation, by hydrochloric acid at 100° C., of free phosphate from casein, from phosphoserine, and from a series of phosphoprotein derivatives, he concludes that phosphoserine is probably the only phosphorised amino acid in the phosphoprotein molecule. It is of course possible that the orthophosphoric acid in the phosphoprotein molecule may be, not singly, but doubly, linked to the rest of the protein. This possibility receives a little support from the fact that casein is not hydrolysed by purified animal phosphatases, which are usually considered to be specific for esters of orthophosphoric acid of the type $R \cdot O \cdot PO(OH)_2$ where R is some alcohol [see Waldschmidt-Leitz & Kohler (21)], whilst the phosphopeptones, and presumably phosphoserine, are so hydrolysed. It is of course equally likely that this difference of behaviour may be bound up with differences in the molecular size of the substrate and not with the degree of esterification of the phosphoric acid. Levene & Schormüller (22) believe that, whereas in vitellin the phosphoserine is not linked to one of the dicarboxylic amino acids, in casein this phosphoamino acid is combined with glutamic acid, in agreement with the somewhat earlier findings of Schmidt (23) and Levene & Hill (24). It may be concluded as reasonably well established that glutamic acid is one of the immediate neighbours in the casein molecule of a large part at least of the phosphoserine.

Linderstrøm-Lang (25) in 1928 described a method by which casein, carefully prepared from cow's milk, could be separated into several different fractions, with different solubilities in dilute (alcoholic) hydrochloric acid and quite different phosphorus contents. In the period under review a similar separation has been effected in order to investigate the action of pepsin on the various fractions, and obtain a further insight into the structure of the casein molecule. Fractions have been obtained by Holter, Linderstrøm-Lang & Funder (26) which vary in phosphorus content from 0.03 to 0.81 per cent phosphorus. A protein having as small a content of phosphorus as 0.03 per cent would not normally be considered to belong to the class of phosphoproteins. Unless some kind of decomposition occurs during the separation process (which at one stage entails heating for 15 minutes at 60°–70° with $N/500$ HCl in 60 per cent alcohol) we must conclude that casein is a mixture of several proteins of slightly different composition, one or more of which

would not normally be described as phosphoproteins. The rate of hydrolysis of the various fractions by pepsin was found to increase with the phosphorus content of the initial material. Linderstrøm-Lang (27) had found the composition of the phosphopeptones, prepared by the action of pepsin on the various caseins of different initial phosphorus contents, to be practically the same. Demanez (28) finds that the caseins separated by Linderstrøm-Lang's method are not immunologically distinguishable by the precipitin reaction.

Stirling & Wishart (29), in reinvestigating the hydrolysis of pure casein by pepsin and trypsin kinase, obtain results which appear to indicate that all the phosphorus of casein is concentrated in one relatively small group in the casein molecule, and do not entirely agree with Linderstrøm-Lang's findings.

Berggren (30) reports that by dialysing milk, contained in collodion membranes, against distilled water, the P:N ratio of the casein separated from the milk decreases progressively. Samples of casein with a phosphorus content as low as 0.29 per cent were obtained. It is concluded that the phosphorus is much more loosely bound to casein than has previously been supposed.

Phosphoric Esters and Carbohydrate Breakdown

For some years past, the attractive possibility that all cells are fundamentally similar in their methods of metabolising carbohydrate has led to numerous comparisons, both experimental and speculative, between the well-known processes of alcoholic fermentation of sugar by yeast and yeast extracts on the one hand, and lactic-acid production from glycogen and glucose by muscle preparations on the other. In the period under review, the part played in both these processes by two phosphoric esters—α-glycerophosphoric acid and phosphoglyceric acid—hitherto not suspected of filling any such rôle, has received considerable attention.

Nilsson (31) examined the products resulting from the action of dried yeast on a mixture of glucose, hexosephosphate, and acetaldehyde, in presence of sodium fluoride. Although no true fermentation could take place under these conditions, the sugar was largely esterified, and a phosphoric ester—monophosphoglyceric acid—having an optically active barium salt, was isolated. On the basis of these findings he made (32) the further suggestion that glyceraldehyde-phosphoric acid, which had been synthesised by Messiner (33) and by Fischer & Baer (34) might play a part in the glycolytic

breakdown of carbohydrate. Embden, Deuticke & Kraft (35) showed that monophosphoglyceric acid is produced when hexose-phosphate is added to a macerated-muscle preparation. Embden suggested at the same time, though he did not actually isolate the compound, that α-glycerophosphoric acid is also present in the reaction mixture, the two esters being simultaneously produced by the "dismutation" of two molecules of either glyceraldehyde-phosphoric acid or dihydroxyacetone-phosphoric acid. The actual isolation of α-glycerophosphoric acid from a reaction mixture in which an aqueous extract of frog or rabbit muscle has acted on glycogen or hexose-diphosphate was accomplished shortly afterward by Meyerhof & Kiessling (36, 37); the α-glycerophosphoric acid found was believed to be the laevo isomer. It would appear that the difficultly hydrolysable phosphoric ester which Lohmann (38) had found to be present in a muscle extract–glycogen–sodium fluoride mixture and Lohmann and Lipmann (39) had separated in an impure form from a muscle extract–hexosediphosphate mixture, and believed to be a carbohydrate-diphosphoric ester, was actually a mixture of phosphoglyceric and α-glycerophosphoric acids. The bearing of these facts on carbohydrate breakdown in muscle and other tissues has been discussed by Embden (35), by Meyerhof (40), and by Nilsson (41).

The once-popular view that methyl glyoxal played an essential rôle in carbohydrate metabolism has been under suspicion for some time, particularly since Lohmann (42) showed that glutathione was a necessary co-enzyme for methylglyoxalase, and that glycogen could be transformed without difficulty into lactic acid in muscle extracts from which glutathione had been removed by dialysis. Embden's new scheme, in which methyl glyoxal plays no part, depends to a large extent on the observations just mentioned, together with the following: (*a*) that simultaneous addition of phosphoglyceric and glycerophosphoric acid to muscle tissue causes an increased formation of lactic acid; (*b*) neither the addition of pyruvic acid alone nor α-glycerophosphoric acid alone leads to lactic-acid production, but if both be added together, twice as much lactic acid appears as pyruvic acid disappears; (*c*) if (synthetic) glyceraldehyde-phosphoric ester is added to muscle tissue, lactic acid is readily formed.

The scheme may be summarised as follows: Hexose diphosphate (or glycogen + phosphate) → 2 mols triosephosphate (glyceraldehyde-phosphoric acid or dihydroxyacetone-phosphoric acid) → 1 mol phosphoglyceric acid + 1 mol glycerophosphoric acid ("dismuta-

tion"). 1 mol phosphoglyceric acid → 1 mol pyruvic acid + 1 mol phosphoric acid. 1 mol pyruvic acid + 1 mol glycerophosphoric acid → 1 mol lactic acid + 1 mol triosephosphate, which again goes through the process described above.

Although the evidence for this hypothesis seems fairly sound, it has not yet been universally accepted, and Embden's scheme has been somewhat severely criticised by Barrenscheen & Beneschovsky (43), particularly his omission of methyl glyoxal from the normal glycolytic path. They state that tissues other than muscle do not produce pyruvic acid from (synthetic) phosphoglyceric acid, and hold that both phosphoglyceric and glycerophosphoric acids are not in the main path of carbohydrate breakdown, but are the products of more or less adventitious stabilisation processes.

Meyerhof appears to have accepted Embden's scheme in the main, and indeed a scheme for the fermentation of hexosediphosphate by yeast somewhat similar to that of Embden for muscle has been put forward by him (40). The difference between alcoholic fermentation and lactic-acid formation is to be found in the fate of the pyruvic acid. While pyruvic acid in muscle extract is reduced to lactic acid by the glycerophosphoric acid, in yeast it is split up into CO_2 and acetaldehyde; the latter is then reduced to alcohol, probably not by glycerophosphoric acid but by the precursor itself, glyceraldehyde-phosphoric acid. Smythe & Gerischer (44) find that synthetic (racemic) glyceraldehyde-phosphoric acid is readily fermented by yeast juice, but only at a little more than half the speed at which the same molecular concentration of hexosemonophosphoric acid is decomposed. With twice the concentration of the triose ester, the speed of fermentation is rather greater than with hexose monophosphate. They find, however, that only half the triose ester is decomposed, the *l*-rotatory component remaining. Actually therefore the *d*-component is somewhat more rapidly decomposed than an equivalent quantity of the naturally occurring hexose monophosphate. Glyceraldehyde itself is only very slowly fermented by the juice. The discovery of phosphoglyceric and α-glycerophosphoric acids as intermediate products, both in lactic-acid formation in muscle and in alcoholic fermentation, appears to have made possible a rather more complete picture of the intermediate steps in carbohydrate breakdown. It is of interest perhaps to note that the new phosphoamino acid, phosphoserine, $NH_2CH(COOH)CH_2 \cdot O \cdot PO(OH)_2$, the recent discovery of which is reviewed in another paragraph, is

very closely related to phosphoglyceric acid, $OH \cdot CH(COOH) CH_2 \cdot O \cdot PO(OH)_2$.

Neuberg & Kobel (45) describe a simplified method for the preparation of phosphoglyceric acid by the addition, at the right moment, of sodium fluoride and acetaldehyde to a fermenting mixture of yeast and either glucose, sucrose, or hexosediphosphate in presence of toluol.

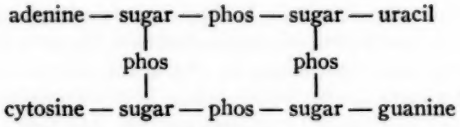
The Phosphatases

Specificity.—Most of the tissue phosphatases will hydrolyse almost any mono-ester of phosphoric acid. Jacobsen (46) and Barrenscheen & Lang (47) have isolated an "adenyl pyrophosphatase" or "adenosine triphosphatase" from liver which seems to be quite specific for the muscle substrate. In fact, muscle adenylic acid (Embden), which is readily hydrolysed by ordinary phosphatases, may be prepared from the triphosphate by the action of the new enzyme. It does not appear to hydrolyse either glycerophosphate or pyrophosphate, and is inhibited, not activated, by Mg ions. The distribution of this enzyme in the organism is quite different from that of glycerophosphatase. Its zone of optimum activity on its specific substrate is, however, in the animal-phosphatase region of pH 8.2–9.4.

From the work of earlier investigators it is known that, if the zone of the optimum pH is in itself any criterion for the individuality of an enzyme, then three glycerophosphatases or perhaps better monophosphatases (capable of hydrolysing the majority of mono-esters of phosphoric acid) exist, one working in a definitely acid range, another at about neutrality or slightly on the acid side of neutrality, and a third in a definitely alkaline range. This has been confirmed by Takahashi (48), who finds also that three pyrophosphatases with similar, but not quite identical, pH zones exist (substrate, diphenyl pyrophosphate). Unlike most animal phosphatases the phosphatase of rabbits' red cells is stated by Hori (49) to have an optimum pH of 4.

Absolute specificity among animal or plant monophosphoric esterases, if it exists at all, must be very rare. Waldschmidt-Leitz & Kohler (21) consider that a specific phospho-amidase is responsible for the hydrolysis of creatine phosphoric acid, but are of the opinion that the existence of a specific phosphodiesterase needs confirmation. The monophosphoric esterases appear to be able to hydrolyse readily phosphoric esters in which only one of the acid hydroxyl groups is

esterified by almost any alcohol. A wide variety of such esters (and pyrophosphoric esters), both naturally occurring and synthetic, are known to be hydrolysed by animal and plant phosphatases (see 3). To these may be added 8-hydroxyquinoline pyrophosphate (50); *o*-, *m*-, and *p*- cresol-phosphoric acids (51); borneol phosphoric acid (52); *l*- and *dl*- difenchyl pyrophosphates (53); mono *dl*-amyl phosphate (54). The existence of specific diphosphoric esterases has been upheld by Takahashi (55), who finds that whilst active monophosphatases do not attack yeast nucleic acid, rapid dephosphorylation is produced when "diphosphatase" and monophosphatase are simultaneously present. He concludes that the four phosphoric esters in yeast nucleic acid are all of the di-ester type, and gives the formula



for this substance. [phos = -O·P(:O)(OH)·O-]

Klein (56) describes the preparation of an active nucleophosphatase, from the intestinal mucosa of the calf, which rapidly attacks animal nucleic acids, but attacks yeast nucleic acid only with difficulty. It is also strongly activated by magnesium salts. Bodansky, Bakwin & Bakwin (57) have found that phosphatase occurs in the cartilaginous, non-ossifying skeleton of the elasmobranch as well as in the dentine-like or bony skeleton of the teleost.

An interesting example of the use of phosphatase as a biochemical tool is given by Myrbäck (58), who finds that by acting on co-zymase with phosphatase, inactivation of the co-enzyme occurs at the same rate as the splitting off of inorganic phosphate. This indicates that the nucleotide isolated from yeast, and believed to be co-zymase, is the activator itself and not merely its adsorbent.

The action on hexosephosphoric esters of the somewhat anomalous phosphatase of the red-blood cells has been investigated by Roche & Roche (59).

Activators and inhibitors.—The specific activation of kidney phosphatases by Mg ions first shown by Erdtman (60) and extended to other animal phosphatases by others (61, 62) has been further investigated by Hommerberg (63) and Pett & Wynne (64). The last-mentioned authors, working with *Clostridium acetobutyli-*

cum (Weizmann) and *Propionibacterium Jensenii* (van Niel) find that the dried organisms in both cases contain an active glycerophosphatase, a hexosephosphatase, and a pyrophosphatase, with an optimum pH varying in each case with the substrate, but within the range pH 5.1–7.2. They find that the hydrolysis of each of these substrates is definitely activated by Mg, giving rather variable q_{Mg} optima with *C. acetobutylicum,* but clear-cut q_{Mg} optima with *Pr. Jensenii* at q_{Mg} 2.5 (q_{Mg} = negative logarithm of the molar concentration of Mg; a similar meaning to that of pH, but avoiding the question as to whether the Mg is present entirely in the ionic form).

Hommerberg (63) finds that $MgCl_2$ in low concentration will by itself catalyse the hydrolysis of monophenyl phosphate and even of glycerophosphate. When enzyme is added, the effect of the two agents together is additive. Lohmann (65) finds that whilst dialysed muscle extract liberates no phosphate from hexosediphosphate, if Mg ions be added, one molecule of phosphate is set free, leaving behind the Embden ester, which, in absence of adenyl pyrophosphate, is not further hydrolysed by muscle extract. The hydrolysis of inorganic pyrophosphate by muscle and liver extracts takes place only in the presence of Mg ions.

The almost universal specific activation of phosphatases by Mg and by practically no other ions has not yet been explained. Kuwabara (66) has reported that the phosphatase of callus tissue is anomalous in being activated by Ca and not by Mg, and the findings of Jacobsen (46) and of Barrenscheen & Lang (47) may be recalled. Takata (67) finds that cholic acid inhibits the phosphatase activity of bone phosphatase both *in vitro* and also when injected into animals. By experimental obstruction of the bile duct, a decreased phosphatase content of the liver and kidney results. Bodansky (68) reports a paradoxical 7 to 23 per cent increase in the phosphatase activity of blood serum on keeping for an hour at room temperature. Yamane (69) finds that potassium arsenate and sodium fluoride have in general a stimulant effect on the activity of bone phosphatase. Pett & Wynne (64) find fluoride inhibitory for bacterial phosphatase. Auhagen & Grzycki (70) have also investigated the action of fluoride on the phosphatases of dried yeast, kidney, and taka diastase. Factors influencing the measurement of phosphatase activity have been investigated by Bakwin & Bodansky (71), in particular the retardent effect of non-protein nitrogenous substances and the accelerating effect of Mg compounds.

Ehrensvärd (72) gives a method for the purification of kidney

phosphatase by the destruction of protein impurities with trypsin and the adsorption of the enzyme on stearic acid.

Phosphatases in tissue.—The possible function of phosphatase in growth and disease of bone has been reviewed by Kay (3), and has received attention in a symposium on ossification (73). The balance of available evidence points to the active participation of this enzyme in the calcification of growing bone, and recent work has not weakened this view. Fabisch (74) has shown that the phosphatase content of normal and of rachitic rat cartilage and also of the cartilage of rats given large doses of irradiated ergosterol, is very nearly the same. This confirms earlier work and the conclusion already arrived at that deficient calcification in rickets is at least not due to shortage of phosphatase in the zone of hypertrophic cartilage cells. The action of the bone phosphatase on the acid-soluble phosphoric esters in the blood and other tissues has been reinvestigated by King (75). These esters in the blood consist of at least two fractions, one being hydrolysed by the bone enzyme at a maximum rate at pH 9, whilst the other is decomposed most easily at a pH of 7 to 7.5.

In avitaminosis-B in pigeons an increase is observed in the rate of phosphorylation by muscle and liver tissue over that in healthy controls, but there is no change in phosphatase activity (76). The intraperitoneal injection of the lymphoid tissue hormone ("P-substance") is said to lead to an increase in the phosphatase concentration in the epiphyseal line in the bones of young rats, and a diminution in the intestinal phosphatase (77). The variation of phosphatase activity in an extract of comminuted whole rat with the age of the animal has been investigated by Kinard & Chanutin (78), who find a maximum phosphatase activity between the thirteenth and the twentieth day of life.

Raw milk contains a phosphatase which has the usual characteristics of a mammalian phosphatase in its pH-activity relationships and its specific activation by Mg ions (79). Kay & Graham (80) find that it is destroyed by pasteurisation rather more slowly than the most heat-resistant of the pathogenic organisms likely to be present in milk, so that its complete absence from a sample of pasteurised milk is a fair index that pathogenic organisms have been destroyed. The same criterion of satisfactory pasteurisation can be applied to cream and to butter made from pasteurised cream.

Tooth phosphatase has been investigated by Mackenzie (81). The distribution and activity of this enzyme (in rats' teeth) are directly

related to the rate of deposition of mineral matter in the tissue. As might be expected, this enzyme appears to be identical in its properties with the corresponding enzyme of bone.

Phosphatase in pathological conditions, etc.—It has been appreciated for some time that a knowledge of the phosphatase content of the plasma or serum may be of value in the diagnosis and treatment of certain types of disease, mainly bone disease (82) [see also O'Reilly & Race (83) for osteitis deformans].

Roberts (84) finds that in obstructive, but not in other types of jaundice, the phosphatase activity of whole blood is increased. Race (85) has described a modified method of plasma-phosphatase determination that he uses in cases of arthritis and osteitis. A micromethod, suitable for use in the clinical laboratory, has been elaborated by Jenner & Kay (86). Bodansky (87) has suggested a number of precautions and corrections to be adopted to ensure the accurate determination of plasma phosphatase. According to Phillips (88) the plasma phosphatase is a good index of the degree of fluorosis in dairy cattle, the enzyme increasing practically in proportion to the level of fluorine intake. Koldajew & Altschuler (89) find a small increase in the phosphatase content of the blood in tuberculosis in man. The increase is greatest in simple (not mixed) tuberculous infections. Smith & Maizels (90) find a high plasma phosphatase in infants suffering from rickets (confirming previous work), which is steadily reduced by suitable therapy. In scurvy the plasma phosphatase rises as calcification of the haemorrhages begins. Smith (91) also finds that on rachitogenic diets, without obvious rickets, the phosphatase is increased. On the other hand conditions characterised by cessation of growth are associated with abnormally low phosphatase. The change produced by a deficient diet in the plasma phosphatase in sheep [Auchinachie & Emslie (92)] provides a much earlier indication of disordered calcium and phosphorus metabolism than either the serum calcium, the blood inorganic phosphate, the general state of health, or the body weight. Diets deficient in Ca (and relatively rich in P) produce a progressive increase in the plasma phosphatase in these animals. Bodansky & Jaffe (93) find that fasted rats and guinea pigs have a much lower plasma phosphatase than fed controls.

The Phosphorus Compounds in the Blood

In a previous review (94) a list was given of the phosphorus compounds which had been demonstrated, or whose existence was sus-

pected, in the plasma or red cells. No new compounds appear to have been added to this list during the period under review. There has, however, been more activity in the investigation of possible changes in the quantity of these compounds in blood in physiological and pathological conditions.

Quantitative changes in blood phosphorus compounds in health and disease.—With regard to the organic phosphorus compounds the reviewer must once more protest against the far too frequent occurrence in the literature of statements as to changes in these phosphorus compounds "in blood" after various experimental procedures, and in various diseases. It is, or ought to be, well known by all working in this field that, apart from phospholipins, the organic phosphorus compounds, i.e., the so-called "acid-soluble organic phosphorus" or "ester phosphorus" compounds, are practically entirely confined to the red blood cells, so that any changes reported "in blood" may merely be evidence of anaemia or polycythaemia, unless the analyses are accompanied by determinations of changes in hemoglobin or in the percentage volume occupied by the red cells, or by other information as to possible blood dilution or concentration.

Stearns & Warweg (95) have filled a gap in our knowledge of the changes which take place with age in the partition of phosphorus in the blood and serum of normal humans from birth to maturity. No phosphorus compound is found to be constant in quantity throughout the entire period of growth. The inorganic phosphorus of serum rises steadily after birth until the maximum value is reached at from four to six months. There is then a slow fall, with approximate constancy from three to twelve years at 5 mg. P per 100 cc. of blood. The fall to the adult value of approximately 3.4 mg. appears to take place rather quickly between sixteen and twenty years. The ester phosphorus in the plasma is highest at birth (1 mg.) and falls to a constant value of about 0.5 mg. after the first month. The lipin P shows a considerable and irregular range of variation (? dietetic). Plasma phosphatase determinations were also carried out and show a low value at birth, rising to a maximum in the first month, and then slowly decreasing throughout infancy and adolescence. In the corpuscles the ester phosphorus rises steadily from birth to a maximum during the second year, then falls slowly to the adult level. The ester phosphorus of the red cells is influenced to some extent by the state of nutrition, children in the best health showing the highest values. Bomskov (96, 97) has investigated the phosphorus partition in the whole blood in humans

from eight to forty years old and in the whole blood of dogs and rabbits of various ages but unfortunately gives no records of cell-volume determinations so that his results lose some of their value. He finds in accordance with Stearns & Warweg that the whole-blood ester phosphorus diminishes with age.

In the phosphatase theory of bone-salt deposition in growing bone (98, 1, 99) a circulating substrate of phosphoric ester is postulated. It is considered that the ester phosphorus of the red cells is probably the main circulating substrate, or store of substrate, for the bone enzyme. The phosphoric ester content of the red cells, according to Stearns, and of whole blood, according to Bomskov, is relatively high during the period of rapid bone growth. This is also true for young rats (100). It is of some interest therefore to find that this substrate is greatly diminished in amount in experimental rickets in rats [Kay (100), Jacobsen (116)] and in rickets in the human [Bakwin, Bodansky & Turner (101)] and is increased in quantity in rabbits' red cells after giving large doses of irradiated ergosterol [Guest & Warkany (102)]. In the rat experiments, the marked diminution in the ester phosphorus could be raised toward normal again by the inclusion of cod-liver oil or irradiated ergosterol in the diet. A similar though smaller change was apparent also in the percentage quantity of acid-soluble phosphoric ester in the liver of these animals. As regards its probable functional activity in bone formation, the bone enzyme in rickets is now seen to suffer from a two-fold handicap. In addition to the shortage of inorganic phosphate in rachitic blood, it is also faced with a considerable shortage of what is presumably the chief substrate for its activity in the circulating blood—the ester phosphorus of the red cells. In Guest & Warkany's experiments, either single large doses, or a series of smaller doses of irradiated ergosterol, given to rabbits, produced a marked rise, both in the inorganic phosphorus of the plasma and in the ester phosphorus of the red blood cells. After some days the quantity of both these constituents began to fall again. Guest & Andrus (103) find that a similar rise in ester phosphorus accompanies the rise in the inorganic phosphate in the blood of dogs with intestinal obstruction.

In the experiments on rickets in which the plasma inorganic phosphorus is low and in the hypervitaminotic conditions and in intestinal obstruction in which the plasma inorganic phosphate is high, it is possible to explain the changes in the quantity of ester phosphorus in the red cells by assuming either (*a*) that a rather slow equilibrium is estab-

lished between the inorganic-phosphate level in the plasma and the ester-phosphorus level in the red cells or (*b*) that the quantity of phosphoric ester in a given red cell is a reflection of the quantity of inorganic phosphate which was present in the circulating blood plasma at the time when the corpuscle was being formed.

Blackwood & Stirling (104), working with lactating and non-lactating cows, find in well-controlled experiments that during lactation, but not otherwise, the "ester phosphorus" of mammary venous blood is definitely lower than that of arterial blood. It is concluded that part of the phosphoric esters of the blood are taken up by the mammary gland during milk secretion, possibly to provide the colloidal calcium phosphate of the milk.

Godden (105) for cows' blood and Mull & Bill (106) for human blood report changes in the calcium and phosphate content at parturition. The former finds that a sharp drop in inorganic phosphate occurs just prior to calving.

Strenuous muscular exercise in man or in the dog does not lead to appreciable changes in either the inorganic or the acid-soluble phosphorus in blood, pointing to a high efficiency in the re-formation of phospho-creatine in the muscle [Gemmill & Ribiero (107)]. Allcroft & Strand (108), however, find that whilst moderate exercise has little effect on the inorganic phosphorus of blood, violent exercise of 15 to 30 minutes duration causes an increase, but the phosphorus figure sinks below the initial value shortly after discontinuing the exercise. Gereb & Laslo (109) discuss changes in the phosphorus compounds of the blood in health and disease.

Probably the lowest inorganic phosphorus yet recorded in blood (0.3 mg. P per 100 cc. plasma) has been found in the so-called beryllium rickets in rats (110, 111). This work throws an interesting side light on the uptake of phosphorus from the diet in relation to rickets. It has been found that on a perfectly satisfactory diet, with or without extra vitamin D in the form of irradiated ergosterol or cod-liver oil, very severe rickets may be brought about in growing rats by the simple expedient of adding 0.5 per cent of beryllium carbonate to this otherwise normal diet. With small quantities of the carbonate down to about 0.1 per cent a milder rickets is produced; beryllium phosphate added to the diet has no such effect. Beryllium forms a phosphate which is insoluble even in fairly acid solutions (pH 4 or less), and its rachitogenic effect is almost certainly bound up with this property, the beryllium dissolved in the gastric juice precipitating quan-

titatively in the intestine with any free phosphate found there and removing it in the insoluble form in the faeces. This provides further support for the view that a chronic defect of phosphorus uptake from the diet is the most important single factor in the production of rickets in rats, and possibly also in other species.

The forms of inorganic phosphate in the serum.—It is well known that only a fraction of the calcium but practically all of the inorganic phosphate of serum is diffusible through semi-permeable membranes. If therefore the "inorganic" phosphate is present partially in a colloidal form it is a colloid which might be described as being maximally atypical. Benjamin & Hess (112) find that finely powdered barium sulphate in excess adsorbs a fraction of the inorganic phosphate, in the form of a filtrable calcium-phosphorus complex of which the quantity appears to be diminished in rickets. The nature of this filtrable, adsorbable calcium phosphate has been further investigated by Benjamin (113), who finds that the complex is also present in artificial solutions containing calcium phosphate and bicarbonate. The epiphyseal cartilage of long bones is found to possess the property of adsorbing the same calcium-phosphate complex, both from serum and from artificial solutions, as is removed by the barium sulphate. This property was found to be limited to the part of the cartilage in which calcification normally occurs; resting cartilage, or non-calcifying rib cartilage failed to adsorb the complex. Faulty calcification in rickets is not due to defect of adsorptive power in the rachitic cartilage, but may be due to a shortage of the adsorbable complex in the blood. This work appears to provide a definite indication that the inorganic phosphate of normal plasma is present in at least two forms, one of which is almost certainly a calcium-phosphate complex, which is very probably of direct importance in calcification. Laskowski (114) finds that, whilst in the non-laying hen the inorganic phosphate of the plasma is almost entirely ultrafiltrable, during the laying period when both the calcium and the inorganic phosphate increase markedly in quantity, only about 50 per cent of the inorganic phosphate is ultrafiltrable, probably as a calcium-phosphate complex. Similar results are obtained after the addition of calcium chloride to beef serum *in vitro* (115).

Owing to shortage of space, a review of recent methods for the determination of phosphorus in various forms of combination will have to be deferred.

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CARBOHYDRATE METABOLISM*

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The present review is based on articles which appeared in 1933. Unfortunately space does not permit a discussion of more than a part of the papers published. Papers which appeared prior to 1933 are quoted with the year of publication in parenthesis, but are not given in the bibliography. The outstanding contribution of the past year in the field of carbohydrate metabolism was the new scheme of glycolysis put forward by Embden, whose untimely death has since been recorded with regret by his colleagues.

Glycolysis in Muscle

Embden, Deuticke & Kraft isolated phosphoglyceric acid as the crystallized barium salt from minced muscle incubated with hexosediphosphate and fluoride. They found that unpoisoned minced muscle converted added phosphoglyceric acid almost quantitatively to pyruvic acid and they thus discovered the immediate source of pyruvic acid in the glycolytic process.

Meyerhof & McEachern found [in confirmation of Case (1932)] that muscle extract formed pyruvic acid from hexosediphosphate or glycogen. Addition of sulfite increased the yield of pyruvic acid and decreased the yield of lactic acid correspondingly. Methylglyoxal formed no pyruvic acid, nor did lactic acid give rise to pyruvic acid, as Hahn (1932) had claimed. Meyerhof & McEachern concluded that pyruvic acid is a normal intermediate on the path to lactic acid.

The occurrence of pyruvic acid as an intermediate of yeast fermentation has long been accepted, and it was shown that the reduced reaction product accompanying pyruvic acid was glycerol, and that both were formed in equimolecular amounts from hexosediphosphate when carboxylase activity was inhibited [Neuberg & Kobel (1930)]. It was recognized independently by Embden and Meyerhof that, in analogy to yeast, the anaërobic formation of pyruvic acid in muscle, which represents a higher oxidation product than sugar, must be accompanied by simultaneous reductive processes. The search for the reduced reaction product led Meyerhof & McEachern to the isolation of α-glycerophosphoric acid, but this was anticipated by Embden *et al.*, who found that addition of phosphoglyceric and α-glycerophosphoric

* Received January 15, 1934.

acid to minced muscle caused the formation of large amounts of lactic acid, while either substance, when added alone, gave only insignificant yields of lactic acid. Embden *et al.* assumed that the pyruvic acid formed by dephosphorylation of phosphoglyceric acid interacted with α-glycerophosphoric acid and that pyruvic acid was thereby reduced to lactic acid, while the glycerophosphoric acid was oxidized to glyceric aldehyde phosphate. They showed further that addition of synthetic glyceric aldehyde phosphate to muscle gave rise to lactic-acid formation and they evolved the following scheme of glycolysis in muscle.

$$\underset{\text{Hexosediphosphate}}{C_6H_{10}O_6(H_2PO_3)_2} \longrightarrow 2\,\underset{\text{Glyceric aldehyde phosphate}}{CH_2OH_2PO_3 \cdot CHOH \cdot CHO} \qquad (1)$$

$$CH_2OH_2PO_3 \cdot CHOH \cdot CHO \xrightarrow{+H_2} \underset{\text{α-Glycerophosphoric acid}}{CH_2OH_2PO_3 \cdot CHOH \cdot CH_2OH} \qquad (2)$$

$$CH_2OH_2PO_3 \cdot CHOH \cdot CHO \xrightarrow{+O} \underset{\text{Phosphoglyceric acid}}{CH_2OH_2PO_3 \cdot CHOH \cdot COOH}$$

$$\underset{\text{Phosphoglyceric acid}}{CH_2OH_2PO_3 \cdot CHOH \cdot COOH} \longrightarrow \underset{\text{Pyruvic acid}}{CH_3CO \cdot COOH} + H_3PO_4 \qquad (3)$$

$$\underset{\text{Pyruvic acid}}{CH_3 \cdot CO \cdot COOH} + \underset{\text{α-Glycerophosphoric acid}}{CH_2OH_2PO_3 \cdot CHOH \cdot CH_2OH} \longrightarrow$$

$$\underset{\text{Lactic acid}}{CH_3 \cdot CHOH \cdot COOH} + \underset{\text{(to reaction 2)}}{\underset{\text{Glyceric aldehyde phosphate}}{CH_2OH_2PO_3 \cdot CHOH \cdot CHO}} \qquad (4)$$

It may be seen that pyruvic aldehyde (methylglyoxal) has no place in the foregoing scheme; its position as an intermediate had been rendered very doubtful previously, notably by the experiments of Lohmann and Embden.[1] Pyruvic aldehyde is now replaced by pyruvic acid. The ability of various tissues to reduce pyruvic acid to lactic acid has repeatedly been demonstrated and dates back to experiments by Embden & Oppenheimer (1912) on the perfused liver. It is significant in this connection that α-glycerophosphoric acid is found in the liver [Fiske (1929)]; diphosphoglyceric acid had been isolated from red corpuscles by Greenwald (1925). Nilsson isolated monophosphoglyceric acid from yeast incubated with fluoride; a simplified procedure for its isolation from yeast was described by Neuberg & Kobel (1). Smythe & Gerischer investigated the rate of fermentation of synthetic glyceric aldehyde phosphate by yeast. They found

[1] See *Ann. Rev. Biochem.*, **2**, 141 (1933).

that only the *d* form was fermented and that the rate of fermentation was equal to that of glucose, while free glyceric aldehyde was fermented much more slowly. These and other isolated observations are now brought under one head by Embden's scheme.

The scheme of Embden was subsequently confirmed in all particulars in Meyerhof's laboratory, where an independent investigation of the same problem was in progress. Meyerhof & Kiessling showed that when the pyruvic acid formed by muscle extract is bound by sulfite, an equivalent amount of α-glycerophosphoric acid accumulates. The α-glycerophosphoric acid was isolated in pure form and identified by elementary analyses. It proved to be levo-rotatory. This is of interest in view of the fact that the lactic acid of muscle, though dextro-rotatory, belongs to the *l* series. A comparison of synthetic and optically active glycerophosphoric acids showed that only the naturally occurring *l*-glycerophosphoric acid was converted to lactic acid by muscle extract.

Meyerhof & Kiessling showed that addition of 1 mol of pyruvic acid and 1 mol of glycerophosphoric acid to unpoisoned muscle extract gave rise to the formation of 2 mols of lactic acid and 1 mol of inorganic phosphate; i.e., the glycerophosphoric acid was also converted to lactic acid by going through the cycle embodied in Embden's scheme. Under the same conditions muscle extract poisoned with fluoride formed only 1 mol of lactic acid and inorganic phosphate was not liberated; instead the glycerophosphoric acid was converted to phosphoglyceric acid. Fluoride did not inhibit the interaction of pyruvic acid and glycerophosphoric acid, but the latter was prevented from going through the complete cycle. This is due to the fact that fluoride specifically inhibits the splitting of phosphoglyceric acid to pyruvic acid, hence the cycle is interrupted at that point and phosphoglyceric acid accumulates. It can now be understood why several workers succeeded in isolating this ester from tissues poisoned with fluoride.

In contrast to fluoride, iodoacetate was found to inhibit the interaction between pyruvic acid and glycerophosphoric acid but not to inhibit pyruvic-acid formation from phosphoglyceric acid. Iodoacetate also inhibits glyoxalase activity by interacting with glutathione which functions as the co-enzyme of glyoxalase (Lohmann). The reaction seems to be a general one for thiol compounds and may be portrayed as follows: $G \cdot SH + ICH_2 \cdot COOH \rightarrow GS \cdot CH_2 \cdot COOH + HI$ (Dickens).

The final step in the confirmation of Embden's scheme in the work of Meyerhof & Kiessling is concerned with glyceric aldehyde phosphate. It was of importance to see whether 2 molecules of the aldehyde would actually yield 1 molecule each of glycerophosphoric acid and phosphoglyceric acid. Evidence was adduced that this is actually the case. In the first place it was found that only one of the optical isomers of glyceric aldehyde phosphate formed lactic acid when added to muscle extract; this is analogous to the results obtained by Smythe in the case of yeast. In the presence of fluoride, glyceric aldehyde phosphate did not form lactic acid and inorganic phosphate was not liberated, but a change had nevertheless taken place; the glyceric aldehyde phosphate which is easily hydrolyzed in acid was converted into esters hydrolyzed only with difficulty in acid. The inference was that the acid-resistant esters formed were glycerophosphoric acid and phosphoglyceric acid, as postulated by the theory, and in order to prove the formation of the former ester, pyruvic acid was added, whereupon lactic acid was formed according to the equation for a fluoride-poisoned extract. When glyceric aldehyde phosphate was added to unpoisoned extract in the presence of sulfite, pyruvic acid was formed, presumably by way of phosphoglyceric acid.

Alcoholic fermentation of yeast has not been investigated completely, but it seems certain that the first steps are identical in muscle and yeast; i.e., hexosediphosphate is changed to phosphoglyceric acid and glycerophosphoric acid and the former yields pyruvic acid. Neuberg & Kobel (2) have shown that addition of phosphoglyceric acid to yeast yields pyruvic acid. The parting of the ways in muscle and yeast is indicated by the further fate of pyruvic acid. In muscle it is reduced to lactic acid. In yeast it is decarboxylated to acetaldehyde and CO_2. According to Meyerhof & Kiessling the acetaldehyde does not react with glycerophosphate but with glyceric aldehyde phosphate. While acetaldehyde is reduced to ethyl alcohol, the glyceric aldehyde phosphate is oxidized to phosphoglyceric acid and the whole process then begins anew.[2]

We see then that fermentation in muscle and yeast is initiated by phosphorylation of the glucose molecule; for this Lohmann's coenzyme system is necessary, which consists of adenosinetriphosphate, magnesium, and inorganic phosphate. The phosphorylized sugar is

[2] While this paper was in press, Meyerhof and Kiessling [*Biochem. Z.*, **267**, 313 (1933)], published a detailed study of the intermediary phases of fermentation in yeast juice.

capable of disruption and for this the presence of the co-enzyme is also necessary, though less is needed according to Meyerhof than for phosphorylation. Hexosediphosphate, by a queer twist of circumstances, is restored to its rôle as "lactacidogen," while hexosemonophosphate must presumably be changed back to hexosediphosphate before it can form lactic acid. Disruption of hexosediphosphate yields glyceric aldehyde, which, unlike the ring forms of the higher membered sugars, behaves like an aldehyde, so that Cannizzaro reactions can set in, which allow the system to go through the same cycle over and over again. According to Meyerhof & Kiessling the co-enzyme system is no longer needed, because pyruvic acid and glycerophosphate can interact with formation of lactic acid in a dialyzed muscle extract. As has long been suspected, the function of the phosphoric-acid group is to render the molecules reactive. Free glycerol, glyceric aldehyde, or glyceric acid cannot replace the respective phosphate esters in the glycolytic system.

The position of methylglyoxal as an intermediary in glycolysis needs to be considered briefly. It will be recalled that Neuberg and co-workers (1929) isolated methylglyoxal as the 2,4-dinitro-phenylhydrazone in almost theoretical yield from specially prepared yeast extracts which they incubated with hexosediphosphate; in mammalian tissues the yield was 20 to 30 per cent of the theory. In view of these results Barrenscheen & Beneschowsky want to retain a direct path from glyceric aldehyde phosphate to lactic acid by way of methylglyoxal and pyruvic acid, apart from the scheme proposed by Embden, but under what conditions one or the other path is followed is not made clear. Since Neuberg's yeast extracts were almost free of co-enzyme, while the dismutation of glyceric aldehyde phosphate depends on the presence of co-enzyme, it is possible that methylglyoxal is merely an abnormal decomposition product of glyceric aldehyde phosphate.

Since lactic-acid formation in muscle is linked with re-synthesis of phosphocreatine, the free energy changes in the various reactions embodied in Embden's scheme and their relation to this re-synthesis should prove to be of considerable interest.

Glucose Absorption

Trimble *et al.* determined absorption of glucose in dogs. The average amount absorbed in grams per kilo per hour was 0.99 for the 1-hour, 1.0 for the 2-hour, and 0.99 for the 3-hour absorption period.

Concentration of glucose varied between 4.5 and 46 per cent and the absolute weight of glucose ingested between 20 and 85 gm., but the rate of absorption observed in the individual experiments showed no definite relationship to these variables. It has been pointed out repeatedly that the blood-sugar curve is not a reliable measure of intestinal absorption and that conclusions as to the rate or duration of absorption, which are based on blood-sugar determinations, have only doubtful value. This is well illustrated in the experiments of Trimble and associates.

The absorption rate of 0.99 gm. per kilo per hour, when compared to the intravenous tolerance rate of 0.9 gm. per kilo per hour as established by Woodyatt (1915) for dogs, shows that there exists a close relationship between the rate at which glucose enters the blood from the intestine and the rate at which glucose can be utilized. A similar relationship has been established for the rat [Cori (1925)]. If the same principle is applied to man, one may infer that the rate of intestinal absorption of glucose can hardly be greater than 0.85 gm. per kilo per hour, because this is the maximum rate at which glucose may be injected intravenously in man without producing glycosuria.

MacKay & Bergman (1) determined the rate of absorption of glucose in rats. Experiments in which a 57 per cent glucose solution was fed showed the following average absorption figures in milligrams per square decimeter of body surface per hour: 1 hour, 101; 2 hours, 78; 3 hours, 78; 4 hours, 74; 5 hours, 73: It may be seen that the absorption rate is considerably higher in the first hour than in the following hours, and MacKay & Bergman state that the greatest importance should be attached to the results obtained for the first hour, because it was chiefly during this period that they observed a relation between the concentration and the absolute amount of glucose fed and the rate of absorption.

Data reported by Horne *et al.* show that the average rate of glucose absorption in mg. per 100 gm. rat per hour was 147 for a 15-minute and 136 for a 30-minute period. They state that this is a negligible difference and that it supports the idea that absorption rates do not fall off with time. Trimble *et al.* also failed to find a higher absorption rate in 1-hour as compared to 2- and 3-hour absorption periods, but MacKay & Bergman (1), in commenting on these experiments, attribute this to the fact that more sugar was fed to the animals killed after 2 and 3 hours than to those killed after 1 hour, while in their own experiments the same dose of glucose was given

for all absorption periods. In support of their contention MacKay & Bergman (1) cite an experiment in which additional sugar was fed each hour in order to prevent a falling off in absorption rate. The averages of this experiment in milligrams per square decimeter per hour for the 1-, 2-, 3-, 4-, and 5-hour period were 81, 66, 77, 79, and 70 respectively; it seems that these figures are not strikingly different from those given in the preceding paragraph. Maddock, Trimble & Carey in replying to this criticism show that when their experiments are grouped in regard to the dose of glucose fed, so that the animals received the same amount of sugar for each absorption period, no support can be found for MacKay & Bergman's contention.

In the same paper Maddock *et al.* report that dogs do not absorb significant amounts of glucose from the stomach and they point out that the activity of the pylorus in regulating the outflow of liquid may be responsible for irregularities observed in the measurements of absorption rates. This is illustrated in the experiments of Horne *et al.*, who conclude that delayed gastric discharge and hence a decreased rate of absorption are responsible for the absence of alimentary hyperglycemia in animals injected with ergotoxine and atropine. Previous workers had assumed that these drugs influence the utilization of sugar, disregarding the possibility that an absorption factor might be involved.

Separate measurements of the amounts of glucose in stomach and intestine, carried out by Trimble *et al.*, showed that an increase in the quantity of glucose introduced into the stomach does not necessarily increase the supply of sugar available for absorption in the small intestine. Ravdin *et al.* determined the concentration of glucose in stomach and intestine of dogs one hour after feeding solutions varying in concentration from 3.5 to 50 per cent. The glucose concentrations in the intestine were very uniform, varying only between 2.4 and 5.3 per cent and Ravdin *et al.* suggest that this may be the explanation for the uniform rate of absorption of glucose in intact animals. In isolated intestinal loops they observed a dependence of absorption rate upon concentration and volume of the sugar solution introduced, but they point out that differences in concentration between 2.4 and 5.3 per cent are too small to influence the rate of absorption.

Other experiments by MacKay & Bergman (2) gave the following results. The actual amounts of glucose absorbed in milligrams per square decimeter of body surface were (average of 3 experiments

each) 1 hour, 201; 2 hours, 228; 3 hours, 246; 4 hours, 296; 5 hours, 355. It may be seen that these animals absorbed 201 milligrams in the first hour, but in the next 4 hours combined they absorbed only 154 milligrams; in other words, absorption practically ceased after the first hour, though there was an excess of sugar present in the gastrointestinal tract at all hours. In two other groups of experiments the animals had actually absorbed less in 2 and 3 hours than in 1 hour: 1 hour, 169; 2 hours, 132; and 1 hour, 241; 2 hours, 206; 3 hours, 204.[3]

Wilbrandt & Laszt attribute the more rapid absorption of hexoses as compared to pentoses to the fact that the former are phosphorylized in the intestinal mucosa, which would increase the gradient of diffusion, while the latter are not phosphorylized. As experimental evidence they offer the fact that iodoacetate inhibits phosphorylation in an extract of the intestinal mucosa and that injection of iodoacetate into rats reduces the rate of absorption of hexoses but not of pentoses. They did not succeed, however, in demonstrating an increased hexosephosphate content of the intestinal mucosa during glucose absorption. Lundsgaard reports that phlorhizin in a concentration of M/200 to M/50 inhibits phosphorylation in minced muscle, in muscle extract, and in yeast, and lactic-acid formation in skeletal muscle, heart muscle, and brain. He points out that with the usual doses of phlorhizin injected into laboratory animals for the purpose of producing glycosuria, only the kidney could contain a sufficient concentration of phlorhizin for an inhibition of phosphorylation. Phlorhizin glycosuria would then be due to an inhibition of phosphorylation and hence of reabsorption of glucose in the tubules of the kidney. In applying these ideas to intestinal absorption Lundsgaard found that M/200 phlorhizin introduced into an intestinal loop greatly reduced the absorption of glucose but not of amino acids. He is therefore of the opinion that esterification is of prime importance for intestinal absorption of glucose. The specificity of phlorhizin action is also shown in Höber's experiments on the kidney in which phlorhizin inhibited the reabsorption of sugars but not of amino acids.

[3] In their paper, MacKay and Bergman (2) expressed the above-described results as amounts of glucose absorbed per hour (by dividing the total amount absorbed by the number of hours); e.g., the last example appears in Table 3 of their paper as follows: 1 hour, 241; 2 hours, 103; 3 hours, 68. This form of presentation does not reveal the unusual nature of the experimental results obtained and there occurs no explanatory statement in the text of their paper.

Wertheimer found in experiments on rats that the intestinal absorption of galactose, glucose, and fructose was inhibited by phlorhizin to a greater extent than that of mannose and dihydroxyacetone, while absorption of pentoses was hardly inhibited. The degree of inhibition is therefore the more pronounced the greater the rate of absorption of a given sugar. The sugars mentioned are all absorbed at the same rate from the peritoneal cavity and on this absorption phlorhizin had no inhibiting effect. Wertheimer's theory is that phlorhizin, by inhibiting phosphorylation in the intestinal mucosa, reduces the rate of absorption of glucose to that of pentoses. The latter are apparently not phosphorylized, nor is phosphorylation involved in peritoneal absorption. If phosphorylation is the determining factor for the intestinal absorption of hexoses, the linear absorption rate of these sugars would find a satisfactory explanation.

Lactic Acid

Cook & Hurst found that blood drawn from the femoral and jugular veins of resting human subjects contained practically the same amount of lactic acid as arterial blood. After moderate exercise (walking) there was no change, but after violent exercise or epinephrine injection the venous blood of the leg contained more lactic acid than arterial blood. Ingestion of 20 gm. of $NaHCO_3$ caused the blood lactic acid to rise 2 to 3 mg. per cent. The authors assume that the normal lactic acid of the blood has its origin in glycolysis of erythrocytes rather than in muscle. Browne & Grant found that minced liver washed free of blood formed less lactic acid than unwashed liver. They attribute lactic-acid formation in the liver mainly to the glycolytic activity of the blood contained in this organ.

Grant observed a rise in liver glycogen in cats injected with ammonium lactate. After removal of the spleen this rise did not occur, but injection of acetylcholine brought back the glycogen deposition from lactate. Hodgson injected sodium lactate in fasted and fed rabbits. He found in both cases a marked rise in liver glycogen, while Bodo & Neuwirth state that such a rise occurs only in fasted but not in fed dogs. Buchwald *et al.* found that during injection of sarcolactic acid in rabbits the extra O_2 consumption accounted for one-fourth of the lactic acid removed; i.e., the oxidative quotient was 4; the optical isomer was removed one-fourth as rapidly and gave an oxidative quotient of 1. Johlin & Moreland reported that turtles recover after 27 hours of anoxia and that they may show blood lactic-

acid values as high as 980 mg. per cent. Schmitt & Cori studied lactic-acid formation in frog nerve. In H_2 and CO at 22° lactic acid accumulated at a rate of about 20 mg. per cent per hour; upon readmission of O_2 it was removed at a rate of 6 mg. per cent per hour. Dickens & Greville showed that addition of lactate inhibited anaërobic lactic-acid formation of brain, testis, and tumor tissues, sarcolactic acid being about twice as effective as its isomer.

Devadatta arrived at the conclusion that all the water in muscle is free to dissolve lactic acid. Resting frog muscle was in equilibrium with a solution containing 30 mg. per cent of lactic acid, fatigued muscle with 238 mg. per cent of lactic acid, corresponding to a concentration of lactic acid in muscle of 16 and 190 mg. per cent respectively. Kerly & Ronzoni determined carbohydrate changes in anaërobic frog muscle kept in Ringer solutions of different pH. At an alkaline reaction lactic-acid increase approximately balanced carbohydrate decrease. As the reaction was made more acid, lactic-acid formation became progressively less while hexosephosphate formation increased, the sum of the two about balancing the loss in total carbohydrate. Ronzoni & Kerly showed that the hexosephosphate accumulated at pH 6 did not disappear when the reaction was changed to pH 9, while lactic-acid formation was resumed at pH 9. Admission of O_2 caused the hexosephosphate to disappear. Wendel (1) showed that dog corpuscles washed free of glucose and in the presence of methylene blue oxidized lactic acid to pyruvic acid and oxyhemoglobin to methemoglobin; the amount of pyruvate formed accounted for the lactic acid which disappeared. In the presence of glucose less pyruvic acid was formed than glucose disappeared, showing that part of the glucose was oxidized by some unknown path.

Heart

McGinty & Miller continued their studies on the lactic-acid metabolism of the dog heart. With the heart intact in the open thorax they found by analysis of arterial and venous heart blood that the average retention was 3.1 mg. of lactic acid and 0.39 mg. of glucose per gm. of heart per hour. The amount of O_2 required to oxidize the retained lactic acid was 44 per cent of the total O_2 consumption. These results suggested that it is lactic acid rather than glucose which furnishes the fuel for the heart. The experiments of Evans *et al.* on heart-lung preparations are in full accord with this conception. When the preparation was ventilated with O_2 containing 6 per cent CO_2,

lactic-acid retention, as calculated from analyses of arterial and venous blood of the heart, amounted to 3.67, and glucose retention to 0.78 mg. per gm. of heart per hour. In previous investigations of this kind the reservoir blood was analyzed and glycolysis of the blood, which would be reckoned as glucose consumption, was not taken into consideration. The authors regard glycolysis of the red blood cells as the source of blood lactic acid and believe that liver, heart, and muscle remove rather than add blood lactic acid.

Cruickshank & Startup emphasize glucose utilization of the heart. They found that the R.Q. of the heart-lung preparation is close to unity and that blood-sugar utilization equals O_2 consumption. During moderate hypoglycemia the R.Q. remained unchanged, but when the blood sugar was almost completely removed, the R.Q. of the heart fell to 0.7 to 0.8. Changes in blood lactic acid were found to be negligible, and blood circulated by a pump, when fully oxygenated, was found to form little if any lactic acid. However, in the experiments of Evans *et al.* blood aërated with 10 per cent of CO_2 in O_2 (pH 7.2) formed 9.4 mg. per cent of lactic acid per hour. These authors point out that, depending on the volume of blood in the preparation in relation to the size of the heart, the uptake of lactic acid by the heart may equal, exceed, or fall behind the rate of glycolysis in the blood. They found a good agreement between the changes in lactic acid in the circulating blood, as actually observed and as calculated from the rate of blood glycolysis, and the rate of lactic-acid uptake of the heart.

Control of Insulin Secretion

For years a large number of experimenters have claimed that insulin secretion by the pancreas is controlled by the vagus nerve, while a smaller number denied the importance of the nervous regulation of insulin secretion. This diversity of opinion continues, but it seems that during 1933 the latter group has decidedly strengthened its case.

LaBarre & Vesselovsky again found that faradization of the right vagus led without exception to a marked fall of blood sugar in chloralosed cats, thus confirming their contention that the vagus stimulates insulin secretion. Geiger heated the carotid blood of dogs under chloralose anesthesia, a procedure which is supposed to stimulate the vagus center. These animals showed a drop in blood sugar and their blood injected into mice caused hypoglycemia; in dogs with the right vagus cut these effects were not observed.

Gayet & Guillaumie repeated the experiments of the workers just mentioned and failed completely to confirm them. Older work of LaBarre (1927), in which the venous blood of the pancreas was transfused into a second animal which showed hypoglycemia upon vagus stimulation of the donor, also failed of confirmation. Furthermore, Gayet & Guillaumie showed that the blood-sugar curves after intravenous injection of glucose were the same in cats with the vagi cut and in intact cats, indicating that the amount of insulin available for the utilization of glucose was the same in both cases. Phillips obtained exactly the same results in unanesthetized cats, some of which were also completely sympathectomized.

That the blood-sugar level itself regulates insulin secretion was shown by Gayet & Guillaumie in the following manner. The pancreas of dogs was transplanted into the neck by means of vascular anastomoses. Stimulation of the vagus nerve of the transplanted pancreas led to copious external secretion, but there was no change of the blood-sugar level. Injection of hyperglycemic blood into the artery of the transplanted pancreas caused a precipitous drop in blood sugar. Similar results were reported by Kosaka, who infused glucose solutions into the pancreatico-duodenal artery, the femoral artery, and the portal vein of decapitate cats. He found that only in the first case was there a pronounced drop in blood sugar, showing that the pancreas responded to an increased sugar content of its arterial blood by liberation of insulin.

Pituitary

Houssay published a summarizing article of the work from his laboratory on the anterior pituitary. When 7 cc. of alkaline extract (equivalent to 1.5 gm. of anterior lobe) were injected daily, dogs developed in 3 to 7 days hyperglycemia, glycosuria, ketonuria, lipemia, and an increased basal metabolism; in thyroidectomized dogs the same symptoms except the increased basal metabolism were present. The symptoms persisted for 1 to 3 days after cessation of injections. Dogs receiving anterior pituitary extracts were very resistant to insulin. Fasted dogs developed neither hyperglycemia nor glycosuria during injection of the extracts. In rats as much as 10 cc. of extract per day caused no glycosuria and only very mild hyperglycemia. Hypophysectomy practically cured the diabetes of previously depancreatized dogs and the animals lived for 6 months. Injection of the extract brought back the diabetic symptoms; in totally as well as partially depancrea-

tized dogs the extract had a much more pronounced effect than in normal dogs.

Evans' results, though similar to those of Houssay, differ in some respects. He finds that in spite of continuing the injections the hyperglycemia, which may be as high as 400 mg. per cent, disappears again after 1 week and that the blood sugar may fall to subnormal values. Glucose-tolerance curves are, however, of the diabetic type even when the blood sugar is normal.

Lucke & Heydemann claim that injection of a commercial anterior lobe extract caused an immediate rise of blood sugar in rabbits and dogs. Injection into the spinal fluid produced a more marked and longer-lasting rise in blood sugar than injection of the same amount of extract intravenously or intramuscularly. In dogs with denervated adrenals the extract had no effect; its action is supposed to be due to a central stimulation of epinephrine secretion.

Benedetto confirmed previous findings in Houssay's laboratory that hypophysectomized dogs are hypersensitive to insulin; anterior lobe extracts diminished this hypersensitivity. Houssay *et al.* found that phlorhizin injections caused in a few days a fatal hypoglycemia in hypophysectomized fasted dogs; these animals excreted less sugar and nitrogen than phlorhizinized dogs with the pituitary intact. Whereas anterior lobe extracts had little effect on normal phlorhizinized dogs, they increased blood sugar, glycosuria, and nitrogen excretion in hypophysectomized and phlorhizinized dogs. D'Amour & Keller observed spontaneous hypoglycemia in hypophysectomized dogs. Hypoglycemic crises were also observed after transverse lesions at the chiasmal level of the hypothalamus, but not after similar lesions in the middle of the hypothalamus. Daggs & Eaton reported an increased sensitivity to insulin and a decreased glucose tolerance in partially hypophysectomized dogs. In experiments of Corkill *et al.* the glucose tolerance of hypophysectomized rabbits was increased and the hyperglycemic effect of epinephrine diminished in comparison to that of normal rabbits. Some of the hypophysectomized rabbits developed marked hypoglycemia after short periods of fasting. The pressor principle of the posterior pituitary did not abolish hypersensitivity to insulin; at the time of hypoglycemic convulsions these animals were well supplied with liver glycogen. Thaddea & Waly found a rise in blood sugar in anesthetized rabbits after injection of the pressor principle of the posterior pituitary; the liver glycogen diminished, whereas the muscle glycogen showed no change. These effects were

also observed in adrenalectomized rabbits. The oxytocic principle had no effect on blood sugar or glycogen.

Epinephrine and Insulin

In spite of the fact that it has been shown repeatedly that killing an animal always causes a considerable loss of muscle glycogen and thereby often leads to erroneous results, this procedure is still widely used in studies on muscle glycogen. Very few investigators take the trouble to ascertain how effective their procedure is in preventing glycogenolysis, though this can easily be done by determining lactic acid in muscle removed for glycogen analysis.

Goldblatt (2) found that epinephrine injections in young rabbits caused a redistribution of glycogen, i.e., there was a decrease in muscle glycogen and an increase in liver glycogen. Insulin also caused an increase in liver glycogen, which suggested the possibility that this might be due to epinephrine secretion evoked by the hypoglycemia, but since the muscle glycogen did not diminish correspondingly, Goldblatt thinks that other explanations are possible. Ergotamine (2 to 5 mg.) inhibited hyperglycemia, but did not prevent the increase in blood lactic acid and the redistribution of glycogen after epinephrine. In an earlier paper Goldblatt (1) reported that, in artificially ventilated cats under amytal or urethane anesthesia, ergotamine inhibited both hyperglycemia and increase in blood lactic acid, which normally follow injection of epinephrine. Sahyun & Webster studied the redistribution of glycogen in rats injected with epinephrine; there was the usual decrease in muscle and increase in liver glycogen. Samson continued his studies on the effect of prolonged intravenous injection of epinephrine. Glycosuria ceased after 24 hours of continued administration of glucose and epinephrine and the author assumes that this was due to a lack of liver glycogen, but glycogen analyses were not carried out to support this contention. Sahyun (1) determined the effect of epinephrine-like substances on the blood sugar of rabbits. A detailed study of blood sugar changes after insulin and glucose in men led Himsworth to the assumption that insulin is activated in the tissues by an unknown factor, before it exerts its effect on the blood sugar. Jonas compared the cutaneous and venous blood-sugar difference in man 1 hour after the ingestion of 100 gm. of glucose and 1 hour after 1 mg. of epinephrine subcutaneously. The average difference was 35 mg. per cent in the former and 14 mg. per cent in the

latter case.[4] Baum *et al.* found that frog muscles kept in Ringer solution containing epinephrine (1 : 100,000) contained less glycogen both under aërobic and anaërobic conditions than control muscles kept without epinephrine.

Gondard found that pancreatectomized dogs with one adrenal extirpated and the other denervated still had severe diabetes. Ring & Hampel showed that the high oxygen consumption of depancreatized cats which they had noted before was still present after inactivation of the adrenal medulla and after thyroidectomy. Canzanelli & Kozody determined the respiratory quotient of exercising depancreatized dogs and concluded that these animals do not oxidize carbohydrate during muscular activity. Yater *et al.* compared the glucose consumption of normal and diabetic heart-lung preparations. The latter consumed only one-fourth as much glucose as the former. They believe that in the diabetic state glucose cannot be used for muscular work. The glucose requirement of a dehepatized dog was estimated to be 0.25, and that of a depancreatized and dehepatized dog 0.19 gm. per kilo per hour. Mann & Bollman showed that insulin accelerates the fall in blood sugar in a depancreatized and dehepatized dog.

Heim & Berg found that lymph collected from the legs of unanesthetized dogs contained slightly more sugar than arterial plasma. Insulin caused a parallel drop of lymph and plasma sugar, with a lag in the increase of lymph sugar during recovery. Ellsworth & Weinstein studied inorganic phosphate changes in serum of normal and adrenalectomized dogs after insulin injection. They conclude that, while the major lowering of blood phosphate after insulin injection may be due to a reflex discharge of epinephrine resulting in accumulation of hexosephosphate in muscle, there is still another mechanism involved which accounts in part for the fall of serum phosphate and which cannot be explained by hexosephosphate formation. This is in accord with the observations by Cori & Cori, who found that glucose plus insulin injected into adrenalectomized and fasted rabbits resulted in a fall in blood phosphate, while insulin alone had only a feeble

[4] Formiguera and Bieto found that injection of epinephrine into the saphenous vein of chloralosed dogs produced no increase in the arterio-venous blood-sugar difference, in spite of the rise in blood sugar. When epinephrine was injected into a mesenteric vein, the rise in blood sugar was accompanied by an increase in the arterio-venous difference. The authors assume that in the latter case epinephrine is destroyed during its passage through the liver, so that it can no longer exert its peripheral effect on muscle of inhibiting blood-sugar uptake.

effect. The latter authors discuss the possibility that during rapid glucose utilization there may be a temporary accumulation of an organic phosphate (other than hexosephosphate) which contributes to the fall in blood phosphate. Davis *et al.* found that the phosphate-lowering effect still occurs to a large extent with insulin solutions which have been treated with acid alcohol or exposed to X-rays and which have lost most of their blood-sugar-lowering effect. Harrow *et al.* found that injection of insulin increases the amount of *p*-acetyl-amino-benzoic acid which is found in the urine after giving *p*-amino-benzoic acid to rabbits.

Nielson perfused the hind limbs of rabbits; addition of insulin increased the rate of disappearance of blood sugar 2 to 4 times but had no effect on disappearance of blood lactic acid. The hexosephosphate content of muscle was not changed by insulin. In contrast to muscle, insulin was without effect on glucose consumption of the perfused liver. Bodo, Cotui & Farba demonstrated glycogen storage in the liver of depancreatized dogs when the blood sugar was raised to high values by glucose injection; insulin accelerated the deposition of glycogen. In normal dogs insulin did not aid in the deposition of liver glycogen and often caused it to fall.

Dixon & Harrison found no evidence for the contention of Hynd (1927) that accumulation of glucosene in the tissues is the cause of insulin convulsions. Freudenberg found, in confirmation of Winter (1926), that *d*-glucal is an antidote for insulin convulsions and causes a rise in fermentable blood sugar; derivatives of glucal, i.e., *d*-hydroglucal and *d*-2 glucodesose, are not antidotal to insulin. According to McCance & Lawrence *l*-methyl-inositol does not raise blood sugar nor relieve insulin hypoglycemia.

Anesthetics

Hall & Sahyun found that in dogs during pentobarbital anesthesia there was usually a slight increase in blood sugar, a moderate fall in liver glycogen, and hardly any change in blood lactic acid and muscle glycogen. According to Banerji & Reid, amytal diminishes the hyperglycemia produced by chloroform, ether, or morphine, but not that produced by epinephrine. Campbell & Morgan's findings are similar: nembutal prevented hyperglycemia after administration of picrotoxine, apomorphine, or ether (by mouth) but not after epinephrine. Phillips & Freeman as well as Banerji & Reid and Ohmi found less hyperglycemia after ether in adrenalectomized than in normal animals. The

conclusion of Banerji & Reid is that epinephrine secretion by the adrenal medulla contributes to the hyperglycemia induced by ether and other anesthetics and that amytal by suppressing epinephrine secretion diminishes the hyperglycemic effect of anesthetics. The "residual" hyperglycemia in adrenalectomized and etherized animals may be ascribed to the direct action of the anesthetic on liver glycogen.

Galactose and Fructose

The blood galactose curve after ingestion of 40 gm. of galactose by normal men was determined by Harding & Grant and by Kosterlitz. Their results are in agreement in showing that the maximal rise amounts to 20 to 40 mg. per cent, but occasionally an increase as high as 60 mg. per cent was noted. The peak of the curve was reached 30 to 60 minutes after ingestion, while after 2 hours usually no galactose was found in the blood. The blood glucose was often found to rise slightly after galactose ingestion. In the experiments of Harding & Grant about 2 gm. of galactose were excreted in the urine, which corresponds to a utilization of 95 per cent.

Kosterlitz & Wedler observed similar blood-galactose curves in diabetics and in normal individuals after ingestion of 40 gm. of galactose. This sugar showed an antiketogenic and protein-sparing effect in normal subjects on a fat-protein diet and in diabetics. When 30 gm. of galactose were given to two depancreatized dogs in which a D:N ratio of 2.7 and 2.6 had been established, 16.8 and 12.8 gm. of extra glucose and 13.3 and 15.8 gm. of galactose respectively were excreted in the urine, corresponding to a recovery of 100.3 and 95.4 per cent of the ingested galactose. It was also found that depancreatized dogs on a meat diet receiving an insufficient amount of insulin did not utilize galactose any better than glucose. Roe & Cahoon found that galactose fed to depancreatized dogs did not cause an excretion of extra glucose, but Bollman & Mann confirmed the results of Kosterlitz & Wedler.

Pollak & Selinger also found a rise in blood glucose after galactose ingestion and similar blood galactose curves in diabetics and in normal subjects. They had previously found however that insulin injections increase galactose assimilation in normal men and in rabbits. In order to explain this apparent contradiction they assume that the high blood glucose in diabetics improves galactose utilization, thus compensating for any dearth of insulin. Pollak & Fehér attempted to find experimental evidence for this thesis. Epinephrine hyperglyce-

mia in rabbits had no effect on intravenously injected galactose, but improved slightly the utilization of ingested galactose. A similar difference was found to exist when hyperglycemia was produced by injection of glucose. This had no effect on intravenously injected galactose, but improved the assimilation of orally administered galactose. Though these results seem rather complex, Pollak & Fehér conclude that glucose improves galactose assimilation.

Sawada found that intravenous injection of glucose, fructose, and mannose in rabbits was followed by a hypoglycemic phase after hyperglycemia had subsided, while after injection of galactose, lactose, and maltose the hypoglycemic phase was absent. A second injection of the same sugar 2 hours after the first injection gave a flatter blood-sugar curve in the case of glucose and fructose but not in that of galactose. Apparently galactose is less efficient in stimulating insulin secretion in the rabbit than glucose or fructose.

It is of importance to emphasize that dogs, rabbits, and rats show a much lower galactose tolerance than man, since they may excrete up to 50 per cent of ingested galactose in the urine. An explanation for this apparent species difference has not been given, and it would seem that caution is necessary in applying experimental results obtained with galactose in laboratory animals to man and vice versa. Interestingly enough, chickens, according to Emslie & Henry, excrete very little galactose, though they absorb this sugar about as rapidly as glucose.

In a comparison of glycogen deposition in the liver from galactose and glucose, carried out on rats and dogs, Deuel *et al.* fed about twice as much galactose as glucose in order to compensate for the loss of the former sugar in the urine. This is a questionable procedure for the following reasons. During absorption of galactose in rats the level of this sugar in the blood is about 230 mg. per cent, while during glucose absorption the blood glucose concentration is about 160 mg. per cent, so that the liver really receives blood with a higher galactose than glucose concentration, in spite of the fact that a large amount of galactose is excreted in the urine. When equal amounts of the two sugars are fed they are offered to the liver for the same length of time because they are absorbed at about the same rate, but in the procedure of Deuel *et al.* galactose is offered the liver twice as long as glucose. Another point is that in comparing the rate of glycogen formation it is necessary to analyze the liver while absorption is still in progress, because as soon as absorp-

tion stops, the liver glycogen begins to diminish again. In their dog experiments Deuel *et al.* fed an amount of glucose which according to Trimble's figures would be absorbed in 2 to 2½ hours; they fed twice as much galactose but made their first glycogen analyses 6 hours after the sugar feeding, i.e. about 4 hours after the end of glucose absorption and perhaps 2 hours after the end of galactose absorption. Between the 6th and 12th hour after feeding, the liver glycogen of the galactose-fed dogs rose from 3.3 to 5.1 per cent. Since Deuel *et al.* did not find a retention of galactose in the tissues, this rise in liver glycogen could not have come from galactose. Between the 6th and 12th hour after feeding, the liver glycogen of the glucose-fed dogs fell from 3.9 to 3.3 per cent and Deuel *et al.* interpret this to mean that galactose is a better glycogen former in the liver of the dog than glucose.

In their main group of experiments these authors maintained rats on diets rich in galactose, lactose, or glucose and determined liver and muscle glycogen after 24 to 72 hours of fasting. At the beginning of the fast each of the three groups of animals contained similar quantities of liver glycogen, but during fasting the rats which had been fed glucose lost their glycogen more rapidly than the galactose or lactose-fed animals. Deuel *et al.* believe that the larger amount of glycogen retained after galactose feeding explains their previous observation that this sugar has a superior ketolytic effect in fasting ketosis of man.

Carpenter & Lee continued their investigations on the effect of glucose and fructose on the human respiratory quotient and the composition of alveolar air. The alveolar CO_2 remained unchanged in trained subjects during the rise in the quotient, but in untrained subjects there was a marked fall in alveolar CO_2. When corrected for the decrease in alveolar CO_2, the maximum rise in the quotient of the untrained subject was the same as that of the trained subject both with glucose and fructose. There was no indication of the production of lactic acid during the metabolism of fructose.

Fermentable Sugar

Somogyi determined the ratio of corpuscle sugar to serum sugar and the rate of glycolysis in bloods of different species. With the exception of man and monkey the corpuscles contained very little sugar and in pigs they were entirely free of sugar. The rate of glycolysis was not related to the sugar content of the corpuscles but ran parallel to the rate of phosphate hydrolysis in the corpuscles [Engel-

hardt (1930)]. Svedberg carried out an extensive investigation of sugar distribution between corpuscles and plasma in different classes of vertebrates. Her paper contains a complete review of the literature.

Kleiner & Halpern contend that blood or plasma kept *in vitro* shows positive and negative variations of its sugar content, but this observation has not been confirmed by Neuwirth. Cori *et al.* determined fermentable sugar in heart and skeletal muscle under various conditions such as glucose, insulin, and epinephrine injections. Short tetanic stimulation caused an increase in fermentable muscle sugar.

West *et al.* gave detailed descriptions for the determination of sugar in urine. All 24-hour samples of human urine contained fermentable sugar, the average in 55 cases being 142 mg. (as glucose). During starvation less fermentable sugar was excreted. Hydrolysis in 0.5 *N* H_2SO_4 increased the fermentable sugar in urine. Various factors affecting the excretion of fermentable sugar were described. When the purified urine sugar was fermented by yeast, the CO_2 production paralleled that from glucose. Harding & Selby, when using another type of copper reagent than in their earlier work, found indications for the presence of small amounts of fermentable sugar in urine of fasting man.

Liver and Muscle Glycogen

Silberman & Lewis found that rhamnose and mannitol formed no liver glycogen in rats, whereas Carr *et al.* found more liver glycogen in rats fed on mannitol plus cacao butter than in controls which received only the latter. Mannitan did not form liver glycogen. In Stöhr's experiments sodium acetate and butyrate formed no liver glycogen; when small amounts of glucose were added somewhat more glycogen was deposited than when glucose alone was fed. The author believes that these results make it probable that the fatty acids are glycogen formers. The possibility that in their presence less glucose is oxidized and more deposited as glycogen is not mentioned. Majer & Reisner found that sodium acetate, butyrate, valerianate, caproate, malate, and citrate formed no liver glycogen, whereas propionate, lactate, and succinate did. Gregg obtained no evidence of a gluconeogenesis from fatty acids in liver perfusion experiments. Higgins *et al.* continued their studies on the diurnal cycle of liver glycogen in the rat and again found it to be dependent on food intake. With the shifting of feeding time the various phases of the cycle were correspondingly shifted.

Hines & Knowlton observed a decrease in the glycogen content of the rat gastrocnemius 3 to 28 days after denervation. Unpublished experiments from this laboratory confirm this observation. In cold-blooded animals, according to Baum & Pichler, denervation causes an increase in muscle glycogen.

Methods

Shaffer & Somogyi gave a description of the properties of various iodometric copper reagents and their use in the determination of glucose and other reducing sugars. No one reagent was equally satisfactory for all sugars, a conclusion also reached by Harding & Downs in a similar study. Harding & Nicholson described several micro-organisms with specific action on certain sugars and their use when dealing with mixtures of sugars. Van Slyke & Kugel recommend the use of Somogyi's zinc filtrate in conjunction with gasometric blood-sugar determinations. A micro-determination of free and combined pentoses is described by Andrews & Milroy. Some of the bound pentoses give low yields in terms of furfural, e.g., adenosinetriphosphate 50 to 53 per cent, muscle adenylic acid 70 to 75 per cent.

Wendel (2) described an all-glass distillation apparatus for the determination of lactic acid and pyruvic acid. As little as 0.07 mg. of lactic acid can be determined with accuracy. Friedemann & Graeser discuss various factors which influence the accuracy of lactic-acid determinations.

Good *et al.* described an abbreviation of Pflüger's method for the determination of glycogen: 1.2 volumes of 95 per cent alcohol are added to the alkaline hydrolysate of tissue and the mixture heated to the boiling-point. This results in immediate precipitation of the glycogen. The charcoal method of Sahyun (1931) was found to give low results, a finding which suggested incomplete hydrolysis of the glycogen adsorbed on charcoal. Bancroft & Fry showed this actually to be the case. Acid hydrolysis of glycogen in the presence of charcoal depended on the concentration of unadsorbed glycogen and acid in the solution, while the adsorbed glycogen was not appreciably hydrolyzed by acid. In replying to these criticisms Sahyun (2) states that addition of 50 mg. of charcoal or less does not lead to a loss of glycogen, while larger amounts cause a loss, as described by the authors quoted above, but Simonovits finds a loss of glycogen even with 50 mg. of Merck's charcoal; however, he apparently did not carry out the purification of charcoal as recommended by Sahyun.

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FAT METABOLISM*

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LIPIDS OF THE BLOOD

A. Normal

Plasma.—Constancy of post-absorptive values. The lipid levels in dogs in the post-absorptive state were studied by Bloor (1) who found that over a period of two years on one diet the level of the lipids remained satisfactorily constant. In the same paper he reports that the post-absorptive level of the phospholipids and often the fat may be disturbed (elevated) for 2 to 3 days after an overfeeding to the extent of adding one-half the maintenance requirement in the form of carbohydrate or fat. Boyd (2) furnishes new data on the various blood lipids of human plasma as the result of analyses in which all the constituents were measured by the one procedure (oxidative). His values for normal plasma lipids are as follows:

	Mg. per 100 cc. Plasma
Total lipid	589
Neutral fat	154
Total fatty acids	353
Phospholipid fatty acids	139
Cholesterol-ester fatty acids	77
Neutral-fat fatty acids	146
Total cholesterol	162
Combined cholesterol	115
Free cholesterol	47
Phospholipid	196
	Iodine number
Total fatty acids	88
Phospholipid fatty acids	124

White blood cells.—Data on white blood cells of normal women are furnished by Boyd (3). As compared with human plasma the total lipid content was four times greater; cholesterol was 50 to 100 per cent greater with about the same amount of combined cholesterol

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but four times as much free; the phospholipid content was four to five times greater than in plasma and twice as great as in red cells. Total cholesterol was the least variable constituent (20 per cent) and phospholipid next at 32 per cent. The iodine number of phospholipid fatty acids was 60, the acids being, perhaps, somewhat more saturated than those of the plasma. White blood cells resemble tissue cells in phospholipid content; cholesterol ester and phospholipid content are reciprocal in their variation.

Lipids and blood proteins.—The lipids in blood have been found by various investigators to be more or less closely combined with the proteins. Wu (4) has examined the effect of these combined lipids on the behavior of serum protein by freeing the proteins of lipids without denaturization, then redissolving the lipid-free proteins and studying their reactions. The proteins were more easily precipitated by sodium sulphate and magnesium sulphate from the reconstituted lipid-free serum than from the original serum. The difference was not due to changes in pH. Other possible explanations and their bearing on the determination of different fractions of the serum proteins are discussed.

Blood cholesterol in the tropics.—The cholesterol level in the blood of natives of India was studied by Ghose (5), who found an average level of about 146 mg. per 100 cc. in whole blood, which is 40 mg. per 100 cc. less than in Europeans or Americans. No significant seasonal variations were observed nor did a vegetable diet have any regular effect.

B. Abnormal

Diabetic lipemia.—Collins (6) finds that increase of serum lipids is associated with glucosuria and acetonuria, but there is no clear relationship between the serum lipid and the blood-sugar level. The lipemia of diabetes may be due to inhibition of carbohydrate metabolism, the mobilization of fat being a response to the caloric requirements of the body.

Curtis, Sheldon & Eckstein (7) report analyses on a case of diabetic lipemia with blood lipids amounting to 14.17 per cent, accompanied by xanthomatosis and lipemia retinalis. The lipids were reduced by insulin and on a high-fat diet the lipemia did not reappear when insulin was given to control the diabetes. Little rise of blood fat occurred with severe glucosuria and hyperglycemia, but production of acidosis by removal of carbohydrate produced an increase of

blood lipids up to 6.4 per cent from which level the administration of insulin caused a fall to 1.08 per cent.

Gibbs, Buckner & Bloor (8), studying the cholesterol/cholesterol-ester partition in the blood of diabetics with severe arteriosclerosis, found the ester-cholesterol percentage of the total cholesterol about 10 points higher (i.e., about 70 per cent of the total cholesterol was combined as compared with a normal value of 60 per cent) than in normals. Since a characteristic of sclerosed arteries is the presence in them of large amounts of cholesterol esters a possible relationship between high cholesterol-ester content of the blood and the occurrence of arteriosclerosis is indicated.

Chaikoff & Kaplan (9) found that, after pancreatectomy in dogs, the cholesterol ester of the blood may entirely disappear. The cholesterol-ester-forming mechanism appears to be in the intestine and involves (*a*) the presence of bile salts, therefore requiring an intact liver [Gardner & Gainsborough (10), Epstein (11)], and (*b*) the simultaneous absorption of fat, therefore requiring fat splitting, which means that the fat-absorption mechanism must be intact. In depancreatized dogs the latter is missing, and the lack of cholesterol esters in the blood may probably be explained on that basis.

Immunity.—The blood cholesterol in immunity or rather in immune serum was studied by Velluz (12). He found that the ratio of cholesterol to cholesterol esters was practically the same as in normal serum. Apparently the phenomenon of immunity is not due to changes in the serum content of cholesterol and its esters. However, as noted in the next reference, cholesterol and especially cholesterol esters may be important in the production of immunity.

Infections. — Marino (13) determined the various lipid fractions in the blood of patients with a number of acute infections, at different stages of the disease. Only in typhoid fever were the changes constant and considerable. During the febrile period of this disease the free cholesterol, cholesterol esters, and phosphatides were below normal. During convalescence there was hyperlipemia with regard to each of these fractions. The results support the theory that these lipid fractions are concerned in the defense mechanism of the body against toxins. Since the changes in the content of cholesterol esters were greatest, these probably play the more important part. Since, as noted in the last paragraph, the immune state is not characterized by low values for cholesterol esters, the decreases in cholesterol esters noted by Marino and by others, notably McQuarrie &

Stoesser (14), in various infections, and also by Okey & Boyden (15) in menstruation, may have to do with tissue changes involved in the initial stages of immunization, in case of menstruation with changes in the uterine surface.

Raab (16) found that in fever, alimentary lipemia is diminished or absent, due, as he thinks, to the increased absorption of fat by the liver.

Eczema.—Hansen (17) finds that the total fatty acids of the blood in infants with eczema have an abnormally low iodine number and that when oils with a high iodine number are fed the eczema improves.

Thyroid.—The effect of thyroid and thyroxin on the level of blood cholesterol has been examined by several workers. Hurxthal (18) finds that blood-cholesterol determination is of distinct value in the clinical diagnosis of hyperthyroidism. Cholesterol is more easily burned in the hotter fire induced by thyroxin.

Turner (19) finds that when whole thyroid gland is administered to rabbits along with cholesterol the changes produced by cholesterol in the aorta are prevented. Thyroxin was found less effective. Potassium iodide also exerts a strong protective action which is not produced by either potassium bromide or potassium chloride. Westra & Kunde (20) found that blood cholesterol, which is higher in cretinous rabbits than in normal rabbits, is lowered by feeding dried thyroid. This change is not correlated with changes in red blood cells and hemoglobin.

Nephrosis.—According to Port (21) an increase in the cholesterol content of the blood is one of the main symptoms of true nephrosis and is present in all stages of the disease. Even after complete cure the blood cholesterol is occasionally high. During the course of the nephrosis a decrease in cholesterol indicates improvement. There is also a rough correspondence between the cholesterol content of the blood and the severity of the edema. Edema first appears with a cholesterol content of 400 to 500 mg. per 100 cc.

Anemia.—Muller & Heath (22) have reported that in anemia due to acute loss of blood the blood lipids remained at normal levels or were high; with chronic loss of blood they were low at the height of the anemia but increased with improvement. In anemic carcinoma of the stomach the blood lipids were low. In chronic myelogenous leucemia the cholesterol was subnormal, while the lipid phosphorus was normal. Aplastic anemia was accompanied by high blood lipids.

Magnesium deficiency.—Kruse, Orent & McCollum (23) discovered that magnesium in the ration at a level of 1.8 parts per million produced a marked increase in cholesterol and especially cholesterol esters in the blood after the fourth week, the increase amounting to about 56 per cent. The per cent of esters increased from the normal 60 per cent of the total cholesterol to 85–90 per cent. Phospholipid was unaffected.

Lipids in the blood of the lungs.—Hoppe (24) tested experimentally the theory of Roger & Binet (80) regarding the destruction of fat in the lungs. He found that the changes in blood fat observed as it passes through the lung were not consistent with this theory. He was unable to find any regularity in the changes in the blood lipids during their passage through the lungs. These findings together with those reviewed earlier[1] would seem to eliminate the lungs as factors in fat metabolism.

Fasting.—In rats fasted to death, Sure, Kik & Church (25) found that blood cholesterol remained constant at 76 to 132 mg. per 100 cc., while lecithin fell from 200 to 150 mg. per 100 cc. Total fatty acids fell from the normal of about 340 to 200 mg. per 100 cc. or lower.

C. Analytical Methods

Nothing essentially new has been put forward for the determination of lipids in blood or tissues either on the macro- or micro-scale. Most of the effort has been in the direction of modifications of existing procedures with the idea in mind of greater ease and accuracy. In the case of fatty acids the tendency in the micro- methods is toward a micro-titration with alkali. For phospholipid, dependence is largely put on the measurement of ether-soluble phosphorus, although it is well known that ether-soluble phosphorus contains other phosphoric acid compounds than are contained in the phospholipids. For the determination of cholesterol the old Tsugaev reaction has been revived.

Total fatty acids.—Smith & Kik (26) determined the total fatty acids of whole blood after extraction, saponification, and extraction of fatty acids by titration with alkali in hot benzene.

Phospholipid.—Kurtz (27) recommends the use of selenium for the determination of phosphorus and nitrogen in the phospholipids, making use of the Kjeldahl digestion. Man & Peters (28) determined cholesterol in blood by weighing the digitonide and determined lipid

[1] Cf. *Ann. Rev. Biochem.*, 1, 284 (1932).

phosphorus by the method of Fiske & Subbarow (81). Katsura and associates (29) modify the chromic-acid oxidative procedure of Bloor (30) for phospholipid in blood so as to use it with 0.2 cc. or less.

Cholesterol.—Milbradt (31) has found the Tsugaev reaction for cholesterol very sensitive and adapts it to the determination of small amounts of cholesterol. Obermer & Milton (32) also make use of the Tsugaev reaction as a micro-colorimetric method and find it satisfactory. Cholesterol is determined gravimetrically by weighing the digitonide in methods offered by Man & Peters (28) and by Ewert (33).

Rappaport & Klapholz (34) measure cholesterol by determining the reducing sugar which can be split off from the cholesterol digitonide, making use of the Hagedorn-Jensen reaction.

Grigaut (35), with a wealth of experience in this field, criticizes the various procedures and recommends a slight modification of the Windaus method. The colorimetric method, making use of the Liebermann-Burchard reaction, has been used by Mirsky (36) for the determination of free and bound cholesterol in urine, the Bloor-Knudson procedure being employed for separation of the two.

Bloch (38) has a good word for the Liebermann-Burchard reaction, although he finds that the color developed in fifteen minutes is due to only ⅔ of the cholesterol present. The determination is justifiable on this basis because the same relative amount reacts. Velluz (37) uses a modification of the Bloor-Knudson procedure for free and bound cholesterol, taking especial care with heating in the evaporation and drying processes.

Schoenheimer & Dam (39) point out that the determination of cholesterol by the digitonide precipitation is subject to rather serious errors. The sterol digitonides were found much less stable and insoluble than previously supposed. They tend to separate into their components or to recrystallize from the solvent. The completeness of precipitation not only depends on the excess of the precipitant used but varies with different preparations of digitonin. The error may be as much as 10 per cent or even greater in certain cases.

The present writer feels that there is as yet no good reason for abandoning the colorimetric method in favor of the digitonin precipitation. Neither procedure is specific for cholesterol and the colorimetric determination is much easier to carry out. Although the results are in general considerably higher (10 to 20 per cent) than those obtained by the digitonin precipitation, they usually run consistently parallel with them. It is not beyond possibility that the lower values

obtained by the digitonin procedure are due to the losses pointed out by Schoenheimer. In a series of determinations on samples of diabetic blood, in which free and bound cholesterol were measured by the two methods on the same samples, Gibbs, Buckner & Bloor (8) found that the colorimetric values for both cholesterol fractions were always 10 to 20 per cent higher than those by the digitonin precipitation but were consistently parallel and the distribution between free and bound cholesterol was practically the same by the two procedures.

PASSAGE OF LIPIDS THROUGH MEMBRANES

Placenta.—The permeability of the placenta for fatty acids and fat has been tested by Chaikoff & Robinson (41) and by Sinclair (42). Chaikoff & Robinson in studying the effects of dietary fat on the fat of the fetus found a relatively small range of effects on the fetal fat as compared with the effect on the mother's fat. The fetal fat ranged in iodine number from 62 to 103, while the mother's fat varied from 36 to 145. There was therefore a transfer of fat across the placenta, but it was limited in extent. The level of protein or carbohydrate in the diet had no effect. Sinclair (42) tested the passage of fat through the placenta in rats in a similar way, that is, by determining the iodine number of the fatty acids in the new-born of mothers which had been fed on distinctive fats during the period of gestation. Having previously found that the fatty acids of the tissue phospholipids were much more responsive to the presence in the diet of small amounts of unsaturated fat than were those of neutral fat, he determined the iodine number of both the phospholipid- and neutral-fat fatty acids of new-born rats and found that feeding cod-liver oil caused a rise in iodine number of the fatty acids of both phospholipid and neutral fat; from which it is quite obvious that the placenta of the rat, like that of the dog and the rabbit, is permeable to fatty acids.

Capillaries.—The postural transudation of lipids through the capillaries of the feet was tested by Man & Peters (43). They found no evidence of diffusion of lipids through the capillary walls. The concentration of lipids, lipid phosphorus, cholesterol, and fatty acids in the blood after a period of standing ran parallel to the concentration of blood protein. Further facts regarding the failure of the passage of lipids through membranes are given in the work of Knauer (79), who found very low values for the lipids in cerebrospinal fluid.

INTERMEDIARY METABOLISM

Change of Fat to Carbohydrate

A notable attempt to furnish information on this controversial question has been made by Murlin and his associates (44 to 47). The attack was made in two ways: first to check up on the transformation of fat to carbohydrate in plants, a change which is generally admitted, and second to obtain evidence on the transformation of fat to carbohydrate in human beings. Murlin made respiratory studies (45) on single castor beans at various times after germination and found respiratory quotients of 0.30 to 0.58, indicating an unquestionable conversion of fat to carbohydrate. The respiratory quotient of the young plant (cotyledons and hypocotyl) alone varied from 0.78 to 100. Therefore, there is in these considerable combustion of sugar. The young plant produces 2.6 times as much CO_2 as the endosperm, whereas it absorbs only 1.3 times as much oxygen. The respiratory quotient of the endosperm alone is low but somewhat higher than that of the whole structure. The combustion respiratory quotient of the beans was studied by Daggs & Halcro-Wardlaw (44). The respiratory quotient of the germinated seed increased with germination, indicating a change from an oxygen-poor to an oxygen-rich substance, probably a change from fat to sugar. The seat of formation of the carbohydrate is the endosperm. Chemical analyses of the beans for fat, sugar, fiber, etc., on all stages up to 250 millimeters hypocotyl length were made by Pierce, Sheldon & Murlin (46). They found a continual decrease in fat and an increase in sugar in the whole seedling up to 40 per cent dry weight at 140 millimeters in length of the hypocotyl, then a decrease of carbohydrate with increase of fiber. The changes in fat and carbohydrate corresponded approximately but the carbon balance was not close. There was apparently an unknown substance formed, possibly an oxidation product of pentose. The respiratory quotient could be accounted for satisfactorily by assuming that two out of six molecules of ricinoleic acid were changed to cane sugar, one to cellulose, and three were oxidized.

Hawley, Johnson & Murlin (47) made respiratory metabolism studies on seven human subjects on high-fat diets and found many respiratory quotients below the theoretical level for the oxidation of fat, a finding which might be interpreted to mean a conversion of fat to carbohydrate. At any rate there was considerable oxygen storage at the time when these low quotients were present. The occurrence of

low quotients depended largely on the tolerance of the subject and tolerance could be acquired and retained for several months. The respiratory quotient was found to bear no intimate relationship to demonstrable ketosis or ketonuria. Correction for ketosis or glycogen formation from protein or glycerol did not change the quotient significantly; neither was the alkaline tide thought significant because there was little acid secreted into the stomach on a fat diet and since there was little protein in the diet there must have been prompt alkali neutralization of the acid secreted. Their explanation of the low quotient was that, in the oxidation of fatty acids, the uptake of oxygen may considerably outrun for a time the production of carbon dioxide and cause a low respiratory quotient. Processes such as desaturation would result in such a condition; so also would β-oxidation preceding the breaking of the chain.

Eckstein (48), testing the transformation of various fatty acids into sugar, found that of butyric, caproic, valeric, and propionic acids fed in different ways, only propionic acid increased the liver glycogen in rats, which is in agreement with the early finding of Ringer (49) that in the β-oxidation of fatty acids having uneven numbers of carbon atoms, sugar is formed from them in proportion to the power to produce propionic acid.

Fatty-Acid Metabolism

The conception of the break-down of the fatty-acid chain at present most widely accepted is that put forward by Leathes (50) and involves a preliminary desaturation of the fatty acids which he assumed to take place in the liver. A doubt as to the ability of the animal organism to desaturate fatty acids is expressed in the work of Burr and his associates. In a paper by Burr, Burr & Miller (51) the statement occurs that "it seems clear that warm-blooded animals synthesize only the saturated acids, and oleic acid, and that they are dependent upon the food supply for linoleic and linolenic acids. One of these two acids must be ingested by the rat if it is to survive."

In work which confirmed this deficiency disease, Sinclair (42) brings forward facts which indicate that a qualification of Burr's statement is necessary. Sinclair points out that in rats raised on a fat-free diet and which eventually suffer from the fat-deficiency disease, the phospholipids contain acids, probably arachidonic acid, which are considerably more unsaturated than oleic acid. Apparently these animals

can form some highly unsaturated acids even though they may not be able to produce linoleic and linolenic acids. It is interesting to note that Burr finds that arachidonic acid does not cure the fat-deficiency disease. It seems probable therefore that different types of desaturation may occur in the animal organism.

On the other side of the question evidence of ability of tissues to desaturate is found in the work of Berend (52) who was able to extract from pancreas with glycerol a dehydrogenase capable of desaturating the fatty acids.

There has never been a satisfactory explanation of why desaturation is necessary as a preliminary step in fatty-acid oxidation. One of Leathes' suggestions was that the double bond made a point of weakness in the molecule and that in the process of metabolism the molecule might break at that point, giving two short-chain acids which could then be more easily utilized than the long-chain acid. Since oxidation of many of the naturally occurring unsaturated acids would yield azelaic acid, Smith (53) tested the ability of the animal organism to burn azelaic acid. It was fed to dogs and the attempt made to recover it in the excretions. It was possible to recover 60 per cent of the acid in the urine, which means that there was little or no utilization of this acid, and the conclusion seems justified that there is no breaking of the fatty acids at the double bond.

Hormones.—Regarding the later stages in the oxidation of the fatty acids, the possible effect of hormones, in particular the hormones of the anterior lobe of the pituitary body, have been studied by Burn & Ling (54) and Deuel and his associates (55). Burn & Ling found that when adult female rats, fed exclusively on fat, were injected with a freshly prepared extract of anterior lobe of the pituitary body there was a striking rise in the excretion of acetone bodies. This result supports the view already advanced that the excretion of acetone bodies on a fat diet depends on the hormones circulating in the blood rather than on any ratio between the carbohydrate and fat present in the diet.

Deuel and associates (55) found that castration produced little effect on the destruction of administered diacetic acid in male rats but in females (in which the excretion had been twice as high as in the male) the operation reduced the excretion to one-quarter that of unoperated female rats, indicating that ketosis was referred to some ovarian hormone. Anterior-pituitary extract increased the acetone-body excretion of normal fasted male and female rats from 1 mg. per

day to 30 to 65 per day. No sex difference was noted in the response. These results are believed to indicate that the active ovarian substance produces anterior-lobe secretion.

Fatty-acid esters.—The nutritive value of various fatty acids fed as esters was studied by Cox (56). The mixed ethyl esters from cocoanut oil were well utilized, but individual esters were never as well utilized as the mixture. Death occurred after feeding: (*a*) ethyl butyrate and caproate, because of refusal to eat; (*b*) ethyl palmitate and stearate, because of inadequate absorption; (*c*) ethyl caprate and laurate, which produced sudden death within two weeks with no definite changes in the tissues. Fatty acids with chains shorter than C_{10} were not stored but were synthesized into higher and more unsaturated acids. Longer-chain fatty acids do appear in the stores and result in a fall in the iodine number of the stored fat.

Examining the utilizability of fatty acids by the chrysalids of the moth, *Galleria mellonela,* Manunta (57) found that cerotic acid will support life in the moths while palmitic will not. Cholesterol was the only sterol found.

The Liver in Fat Metabolism

Leathes' theory of fat utilization involves the probable formation of phospholipids from fat in the liver. The experiments of London (58), who studied the blood entering and leaving the liver during fasting, led him to the conclusion that the stored fat of the organism was carried to the liver and there changed over into phospholipids, a finding which is in agreement with Leathes' ideas. The evidence involving the liver in the production of phospholipid has, however, not been large in amount or very satisfactory in character, the difficulty apparently being that when phospholipids are formed from fat in the liver they are so promptly removed that it is difficult to find them either in the liver or in the blood leaving the liver.

Artom (59) succeeded in getting positive evidence as to the formation of phospholipids by the liver by feeding very large quantities of neutral fat to dogs and examining the phospholipid content of the liver. He found under these conditions increases of the liver phospholipid up to 36 per cent, and since the proportion of fatty acids in the phospholipids was in agreement with the ordinarily accepted formula, true phospholipids were formed. His conclusion was that the phospholipids have an important rôle in fat metabolism.

The effect of various substances on the lipid content of the liver

has continued to attract attention. Blatherwick and associates (60) found that, when fed whole liver, rats developed livers containing much fat and cholesterol esters. The cholesterol of the liver itself is one of the effective substances bringing about this change, but there is another factor which is probably more important. It is soluble in water and 70 per cent alcohol. The fat accumulated in the liver is normal liver fat. The livers are larger and the percentage of solids is higher. Lecithin has no effect on fatty livers caused by feeding liver.

Chanutin & Ludewig (61) found, after feeding cholesterol to rats, an accumulation of fat and cholesterol esters in the liver and it was the only organ showing great changes. Fat, cholesterol, and cholesterol esters progressively increased, while phospholipid remained normal. The relative weight of the liver increased. Carbohydrate fed along with cholesterol produced a greater effect on esters and total lipids than when fat was fed.

Meier & Thoenes (62) studied the respiration of the fatty liver produced in guinea pigs by chloroform and phosphorus poisoning, making use of the Warburg apparatus. They found the respiration at least 50 per cent greater than normal and with a low respiratory quotient. Thoenes (63) found that the fatty liver caused by phosphorus poisoning in guinea pigs does not form lactic acid from carbohydrate to a greater extent than normal liver, nor is the synthesis of carbohydrate from the lactic acid impaired.

Unsaponifiable fraction.—The "unsaponifiable" fraction of mammalian liver has been studied by two groups of workers. Fränkel & Löhr (64) found in the unsaponifiable fraction from liver a yellow oily substance not precipitated with digitonin but reacting to the Liebermann-Burchard and Salkowski reagents. It could be distilled in high vacuum at low temperature and is probably an alcohol. The liver contains 0.02 per cent of lignoceryl-sphingosin and 0.03 per cent of polydiamino-phosphatide. The cholesterol-free unsaponifiable substance amounted to 0.1 per cent of the moist weight and contained 20 per cent of sphingosine, which gave the extract an alkaline reaction and which could be precipitated as sulphate with alcoholic sulphuric acid.

Freytag & Smith (65) found that sterols constituted about 64 per cent of the total unsaponifiable fraction of liver. Dehydrocholesterol and ergosterol were present in small amounts, and lignoceryl-sphingosine was identified. Vitamin A was found in relatively large proportions and vitamin E in minute traces.

Cholesterol Synthesis and Destruction

It is now generally admitted that cholesterol can be synthesized in the animal organism. Schoenheimer (40) has also established the probability of cholesterol destruction in humans by a study of a case of hypercholesterolemia.

Experimentally both synthesis and destruction have been shown in a very striking way in the same animal by the work of Schoenheimer & Breusch (66). They confined mice in large flasks and fed them with various substances over a period of a month, after which the animals were killed and the carcasses with all food residues and excreta were digested in the flask and the changes in the cholesterol studied. With bread alone the mice synthesized in one month as much cholesterol as they contained initially; with the addition of moderate amounts of cholesterol to the food a considerably smaller amount was synthesized, and, when large amounts of cholesterol were given, a considerable portion was destroyed. Fat or carotene had no effect on the cholesterol balance. Bile acids increased the destruction of cholesterol, probably because of better absorption from the intestine. On bread alone the average synthesis per mouse per day was 1.8 mg.; with bread and fat, 2.1 mg.; with bread, lard, and 200 mg. of cholesterol per month the synthesis was 1.1 mg.; with bread, lard, and 1300 mg. of cholesterol per month the destruction was 4.4 mg. Synthesis of cholesterol therefore takes place when necessary, and when present in excess it is destroyed.

Kaufmann & Mühlbock (67) studied the cholesterol metabolism in pregnancy and lactation. They found that in spite of the hypercholesterolemia and the requirements of the fetus, the cholesterol balance of pregnant women is negative. Even the large demand for cholesterol in the lactation period is accompanied by an excess of fecal cholesterol over that ingested.

Fat-Deficiency Disease

A phase of the fat-deficiency disease in which carbohydrate is changed to fat to an abnormal extent is discussed by Wesson & Murrell (68). The known vitamins and amino acids are not effective for its cure. Linolic acid is not effective, although it cures Burr's deficiency disease. Lard contains the corrective factor, which is acid in nature and is resistant to alkali. The curative substance is apparently separated with the liquid fatty acids since it requires only 0.7 grams per

100 grams of body fat of the liquid acids to protect, while it requires more than ten times as much of the solid fatty acids.

The Lymph in Fat Mobilization

Rony, Mortimer & Ivy (69) have found that while the thoracic duct lymph of dogs, 24 hours after a fat meal, contains only 0.2 of 1 per cent of fatty substances, the fasting lymph (2 to 14 days) contains 0.25 to 1.3 per cent (average 0.63 per cent) total fatty acids, the higher values being found in young dogs. The chief source of this extra fat is the intestine because when the intestine is removed there is no increase in the fat of the lymph in fasting. Fat is apparently secreted into the intestine. Part of it is excreted, constituting the endogenous-fat excretion, one of the purposes of which appears to be to carry off excreted sterols. The remaining fat secreted into the intestine is apparently digested and absorbed in the regular way. These findings involve the intestine in the metabolism of fat in fasting. The remaking of the fat by the intestine implied by these findings is in line with the desaturation of the fat by the intestine noted above, and with the earlier findings of Bloor (70) and McClure & Huntsinger (71) that the fat which reaches the blood by way of the thoracic duct during fat absorption may be considerably different from the fat being absorbed.

MISCELLANEOUS ARTICLES

Fat of Milk; Butter Fat

Eckstein (72) has determined the amounts of linoleic and linolenic acid in butter fat from the weights of the bromine fraction. Linoleic acid varied from 0.17 to 0.25 per cent, linolenic from 0.07 to 0.17 per cent. The content of linolenic acid could be increased by the addition of linseed meal to the ration, which means that the content of this acid in milk fat is apparently dependent on the diet.

An extensive study of the fatty acids of butter fat has been made by Bosworth & Brown (73). They found acids of the saturated series from butyric (C_4) to lignoceric (C_{24}) and possibly higher. The lower acids, they think, may be the result of beta oxidation of the higher fatty acids in the normal metabolism of the mammary gland. They found among the unsaturated acids, decenoic, tetradecenoic, tetracasanoic, and other unsaturated acids higher than oleic but no linolic. The presence of palmitoleic and gadoleic acids was doubtful.

Lipids in Plants

Jordan & Chibnall (74) have made an important contribution to the physiology of the lipids in their studies on the runner bean. All the organs contain lecithin and cephalin, and in progressively smaller amounts from the cotyledons to the pinnate leaves. The cotyledons and embryo axes also contain a small amount of magnesium phosphatidate. On germination the relative amount of this salt in the growing organs increases rapidly until the prophylls start to expand when the magnesium is slowly replaced by calcium. Calcium phosphatidate is the chief phosphatide of the mature prophylls and pinnate leaves. In germination the disappearance of phosphatides from the cotyledons is much more rapid than the appearance of phosphatides in the growing organs. The seed phosphatides are believed to function chiefly as reserve food. In starvation experiments leaf phosphatides disappear slowly and hand in hand with protein. It is therefore probable that they are integral parts of the protoplasm. In starvation experiments the waxes and unsaponifiable material remain unchanged, thus confirming the view that they are end-products of metabolism.

Diphtheria Bacillus

Chargaff (75) examined the lipids of the diphtheria bacillus. He found no glycerol either in fat or phospholipid. Instead there was a polysaccharide in fat and an aldohexose in the phospholipid. The saturated fatty acid was mostly palmitic and the unsaturated acid was palmitoleic, with the double bond in the 8–9 position. These peculiarities in the composition of familiar lipids are in agreement with those previously recorded by Anderson and his associates (many recent papers in the *Journal of Biological Chemistry*) and indicate that the lipid metabolism of bacteria may be quite different from that of higher forms of life.

Lipids of the Gums

The phospholipid and total lipid content of the gums, according to the work of Hodge (76), lie between the values reported for skin and for the intestinal mucosa. There is no correlation between the lipid content and the health of the mouth.

Brain

The lipid-sulphur compound of the brain has been isolated by Blix (77) and found to be a cerebron–sulphuric-acid ester. The isolated

substance contained potassium and the compound was probably the potassium salt. It is apparently the only sulphur-containing lipid of the brain and makes up about a quarter to a fifth of the total brain cerebroside.

Fatty Acids of Starch

It has been known for a long time that it is very difficult to extract the fatty acids from starch and it has been believed therefore that the fatty acid must be in combination in some way with the starch molecule. Taylor & Sherman (78) found that the fatty acids in corn starch occur entirely in the alpha-amylose or insoluble portion, the soluble beta-amylose being pure carbohydrate. The fatty acids are not extractable with solvents but are set free only after long hydrolysis with acid or with lipase-free amylase. The acids, which consist of palmitic, oleic, and linolic, are liberated preferentially, the unsaturated acids coming off first.

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THE METABOLISM OF PROTEINS AND AMINO ACIDS*

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The reviewer has laid special stress upon the literature of amino-acid metabolism which appeared up to September, 1933. Consequently that on protein metabolism which was published during the same period has been relegated somewhat to the background.

Ammonia

Ammonia is formed from casein during tryptic and peptic digestion. Its formation is especially pronounced at the beginning. Ammonia formation by kidney extract is not marked. However, amidolysis and aminolytic activity are distinct (1).

The stomach contains 2 to 9 mg. per cent of ammonia derived from the urea of the blood (2).

Wassermeyer & Rohrbach (3) investigated the ammonia content of the heart in the case of an alteration of the heart muscle resembling myocarditis. They also studied post-mortal ammonia formation.

Two different processes of ammonia formation in sheep's blood have been investigated. One takes place in intact blood cells, while the other proceeds only after haemolysis. The latter is inhibited by borate (4).

When ammonium carbonate is injected into peripheral veins the ammonia content of the kidneys is found to be higher in the veins than in the arteries (5). The same holds true for the pancreas.

Urea

Urea formation in the liver of mammals as reported by Krebs (6) is found to take place also in amphibia and reptiles. Among fish only those containing arginase exhibit urea formation in the liver. Negative results were obtained with birds (7). Tissue slices of liver and kidney on immersion for 3 hours in physiological salt solution of pH 7.4 at 37° C. can form ammonia and urea from added amino

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acids. Under such conditions arginine causes much urea formation. Brain and embryonic tissues as well as tissues from tumors and anaërobic glycolytic tissues are inactive in this respect (8).

Plants containing urea, and also plants which do not contain it, resorb arginine as such from the roots and break it down later on in the plant (9).

Amino Acids

Glycine.—Glycine administration in cases of muscle atrophy or pseudohypertrophic muscle atrophy increases the creatine content of the urine during a few weeks, returning then to normal. As the creatine content of the urine decreases the creatinine output increases. Clinical improvement, objective and subjective, follows the administration of glycine. In amyotrophic lateral sclerosis, severe arthritis, and congenital idiocy, glycine is without effect. The creatine output also does not increase (10).

From determinations of urinary nitrogen and carbon it appears that starved rats assimilate glycine imperfectly with respect to nitrogen. The assimilation of an amino-acid mixture is, however, imperfect with respect to carbon (11).

Alanine.—On perfusion of the stomach *d*-alanine and *dl*-alanine form *d*-lactic acid (12).

Leucine.—Under the same conditions *l*-leucine forms acetone, acetoacetic acid, and β-hydroxybutyric acid (13).

Citrulline.—Wada isolated this amino acid from watermelons for the first time and obtained it later as a product of the digestion of casein (14). On treatment with acids, citrulline forms proline. Alkaline treatment yields ornithine (15). *B. pyocyaneus* changes arginine to citrulline (yield 4.9 per cent). This conversion is brought about by an enzyme—arginine desaminase. *B. coli* does not form citrulline under the same conditions (16).

Arginine.—On injecting various amino acids into the hen's egg, glycine, alanine, leucine, phenylalanine, and tryptophane exert a diminishing influence upon creatinine formation, while tyrosine, glutamic acid, serine, histidine, carnitine, and especially arginine act as promoters (17).

The nutrition of the embryo is independent from the catabolism of the mother. During infancy the arginine content is high (18).

Tyrosine.—In rabbits tyrosine hydantoin combined with glucu-

ronic acid is eliminated in the urine. The potassium salt of tyrosine-hydantoin-glucuronic acid was obtained in the crystalline form and analyzed (19). Tyrosine-thio-hydantoin can combine in the organism with sulphuric acid and glucuronic acid, but tyrosine and tyrosine-uramino-acid will not combine (20).

The phosphoric-acid ester of tyrosine may be synthesized by esterification with formic acid and displacement of the formic acid radical by phosphoric acid (21). Tyrosine and phenylalanine on oxidation with H_2O_2 in the presence of $FeSO_4$ yields 3,4-dihydroxy-tyrosine and tyrosine respectively (22).

B. coli attack tyrosine with formation of hydroxy-phenyl-lactic, hydroxy-phenyl-propionic acid, or tyramine, depending upon the strain of bacteria used. There are, however, certain strains which do not form any of these acids (23).

Five minutes after injection of 5 gr. of tyrosine into the jugular vein, 95 to 98 per cent of the tyrosine disappears from the blood, the amino-nitrogen increases a little during the first hour (4.7 mg. per cent), and the urea nitrogen remains unchanged. Small amounts of tyrosine appear in the urine. Extirpation of the thyroid glands has no influence (24).

During the hibernation of toads the leucine, alanine, and valine content of the liver increases. The latter contains considerable tyrosine during the summer (25).

Histidine.—Kiyokawa obtained relatively much urocanic acid in the urine of rabbits after injection of *l*-histidine hydrochloride (26). On using *dl*-histidine for the same experiment he isolated *d*-histidine instead of urocanic acid. When histidine was broken down by the aid of *Oidium lactis,* imidazole-propionic acid was obtained.

Histamine.—By the action of histaminase upon histidine, CO_2 is evolved with absorption of oxygen. Histaminase occurs in the kidneys and in the intestines but could not be detected in the brain, in the spleen, or in the lungs (27).

Histamine appears in the stomach on perfusion (28).

Histamine and other putrefactive products of protein increase the fat content as well as the lipoid content of the blood. Preparations from the thyroid gland and adrenaline, however, lower the percentage of fat and lipoid in blood (29).

After having been treated for 40 days with a daily injection of 1 mg. of histamine, rabbits become more susceptible toward histamine injections than the normal untreated rabbit (30).

Histamine injections of 0.7 mg. per kg. lower the blood-sugar content for a few hours (31).

Histamine and adrenaline form a compound with gelatine. Histamine is able to partially displace adrenaline from its protein combination (32).

Tryptophane.—Kotake and collaborators have published their second report on intermediary tryptophane metabolism (33). The formation of anthranilic acid from tryptophane under the influence of *B. subtilis* proceeds through kynurenin (34). The presence of kynurenin as an intermediary product could be proven; it was also shown that kynurenin is converted to anthranilic acid by *B. subtilis* (35). Kynurenin is found in normal urine (36).

If *Willia anomala* is cultivated in a liquid containing tryptophane a compound results in which the acetaldehyde formed is combined with tryptophane (37). Indole-pyruvic acid and *dl*-indole-lactic acid are converted into indole by *B. coli*, but *l*-indole-lactic acid remains unchanged (38). This fact seems to indicate that *l*-indole-lactic acid corresponds to *l*-tryptophane as far as its configuration is concerned (39).

Aminoacetophenone, which is a cleavage product of kynurenin in decomposition *in vitro* (40), combines with glucuronic acid in rabbits. Previous to this combination aminoacetophenone is reduced in the animal body to *o*-aminophenyl-ethylcarbinol (41). Tryptophane solutions become colored upon prolonged heating after the previous addition of a little hydrochloric acid and an excess of Lugol's solution. The colored product is soluble in amyl alcohol, and the alcoholic solution shows an absorption band between D and E, close to E (42).

Rats utilize subcutaneously injected tryptophane for nutrition (43) contrary to Jackson's results (44). Kynurenin cannot replace tryptophane in nutrition (45). It accelerates, however, the growth of yeast cultures (46).

Tryptophane, on heating to 100° C. with ethyl chloride in a sealed tube, yields *dl*-N-N-ethanyl-tryptophane (47). *dl*-tryptophane may be separated into the *d* and *l* forms by treatment of the quinine salt of acetyl-tryptophane (48).

Tryptophane, tyrosine, histidine, leucine, and lysine are essential for the growth of *Aspergillus niger* (49).

The following rates of absorption of tryptophane derivatives, in mg. per 100 gm. body weight per hour, are reported for rats (50).

Acetyl-*dl*-tryptophane, 91.8; acetyl-*l*-tryptophane, 86.1; *l*-tryptophane, 62.9; *dl*-tryptophane, 57.9; ethyl ester or *dl*-tryptophane, 40.4; and ethyl ester of *l*-tryptophane, 37.0.

Amino acids containing sulphur.—Cystine may be replaced by methionine in the food of rats (51). By the action of sulphuric acid on methionine the disulphide homocystine is formed (52). It has the formula:

$$\begin{array}{l} S-CH_2-CH_2-CHNH_2-COOH \\ | \\ S-CH_2-CH_2-CHNH_2-COOH \end{array}$$

Homocystine may be fed to rats instead of cystine in quantities of 58–66 mg. per day (53). *dl*-methionine is absorbed somewhat less readily than *l*-cystine. In this case the urine gives positive tests for the —S—S— group (54).

Cystine is not catabolized in the animal body as easily as other amino acids (55). It is the only sulphur compound in the membrane of the hen's egg (56). On heating or by aëration for one year it is oxidized to cysteic acid (57).

Deamination.—Warburg (58) has studied the deamination of amino acids by charcoal in the presence of oxygen, and Baur has investigated the same reaction in the absence of oxygen (59). Baur claims that glycine can be deaminized hydrolytically. Wunderly substantiated Baur's claim by his work on alanine, leucine, and aspartic acid (60). Wieland, however, is of the opinion that Baur's and Wunderly's results are due to oxidation, brought about by the oxygen of the charcoal (61). Baur and Wunderly have repeated their experiments and rigorously excluded oxygen by working in inert gases (H_2, N_2, CO_2) and also in water vapor (62). They proved that alanine may be deaminized by hydrolysis (63). Aspartic acid was split in the first phase of the reaction according to the following equation:

$$HOOC-CHNH_2-CH_2-COOH \rightarrow HOOC-CHOH-CH_3+NH_3+CO_2$$

In the second phase malic acid and pyruvic acid are formed (64, 65). The heats of reactions for both processes were also calculated (66).

This deamination is brought about not only by charcoal but also by charcoal extracts (67). In using extracts of charcoal, glycine,

alanine, and aspartic acid are deaminized to a less extent than by charcoal. With phenylalanine, however, the deamination is the same in both cases (serine 0.2 per cent; sarcosine 0.05 per cent; *dl*-glycylglycine 40 per cent; *dl*-leucylglycylglycine 8 per cent) (68). This type of deamination also takes place after the charcoal has been poisoned by H_2S and HCN. It also works in the presence of phenylurea and with other surface-active substances (69).

According to Bergel & Bolz, amino acids undergo autoxidation in the presence of active charcoal (70). Peptides, diketopiperazines, amides, and betaines are stable but the alkyl amino acids are easily oxidized. With kidney slices the reverse is true.

Krebs has studied the cleavage of amino acids *in vitro* by living tissue slices of different animals (71). He found that the deamination of amino acids proceeded in the liver and in the kidneys, and was especially intensive in the latter. This seems to be important in the regulation of the acid-base equilibrium. Alanine, valine, leucine, phenylalanine, and histidine were easily deaminized by the kidney slices. Deamination of the *dl*-amino acids proceeded more rapidly than the deamination of the natural amino acids $\left(\frac{1}{10} - \frac{1}{20}\right)$. α-amino-dicarboxylic acids had the highest rate of deamination among the natural amino acids. In the case of dipeptides it is not clear whether the deamination preceded the peptide splitting or vice versa.

The deamination of the amino acids in the organism is an oxidative process and the amino acids are converted to keto acids. Pyruvic acid, α-keto-butyric acid, phenyl-pyruvic acid, and α-keto-isocaproic acid were isolated as the dinitro-phenylhydrazones. As a rule the deamination of amino acids is bound up with oxygen consumption and does not occur in a medium free from oxygen.

An enzyme, amino-oxydeaminase, occurs in the kidney and diffuses from the tissue slices into the physiological salt solution. This enzyme also exists in the liver in small quantities, but it is not found in any other organ. It is not poisoned by HCN and As_2O_3.

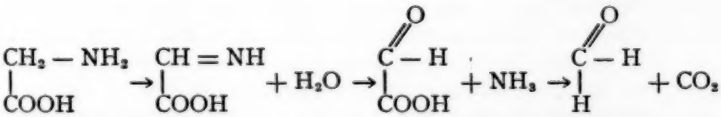
The keto acids resulting from the deamination of aspartic acid and glutamic acid undergo a further degradation (72, 73). By means of As_2O_3 one obtains at first keto-glutaric acid and then oxalacetic acid.

dl-phenylalanine is easily deaminized by the animal body. The resulting phenyl-pyruvic acid is excreted by the kidney (74, 75).

Kotake and associates have worked out a method for the determination of small quantities of hydroxy-phenyl-pyruvic acid and hydroxy-phenyl-lactic acid in urine (76). With the aid of this method they have studied the degradation of tyrosine in the animal body. They found that even 1 gr. of *dl*-tyrosine increases the concentration of the keto acid in the urine. In the case of *l*-tyrosine 5 gr. are necessary to bring about the same result. Hydroxy-phenyl-lactic acid and hydroxy-phenyl-pyruvic acid are mutually interconvertible in the organism. Shortly after feeding *l*-tyrosine one finds an increase in the hydroxy acids in the urine. If the *dl*-form is used instead, one discovers, however, chiefly hydroxy-phenyl-pyruvic acid in the urine. The same relation was found in perfusion tests on liver and kidney. Kotake and associates are of the opinion that the physiological degradation of tyrosine involves the formation of hydroxy-phenyl-lactic acid as the main intermediary compound. The formation of this intermediary compound is probably due to hydrolysis as illustrated by Wunderly's charcoal experiments (63).

According to Bergmann, glycyl-dehydro-phenylalanine is split by an enzyme in the kidney, glycine, ammonia, and phenyl-pyruvic acid being the products (77).

Catalytic deamination of glycine and serine with pyro-catechol, hydroxy-hydroquinone, and adrenalin as catalyzers yields CO_2 and NH_3 in the ratio of 1 : 1. Glycyl-tyrosine and glycyl-glycine are deaminized without the evolution of CO_2. Alanine, leucine, and isoleucine form only a small quantity of CO_2 and NH_3 (78). If methyl glyoxal is used as catalyzer the deamination is somewhat different (79). Barrenscheen & Danzer (80) obtained glyoxylic acid from glycine on employing pyrocatechol. Edlbacher found that glycine on deamination with adrenaline yields a small amount of formaldehyde (81).



Blasche is of the opinion that the mode of deamination of amino acids in the organism is at the present time still purely hypothetical (82).

Changes in Amino Acids on Ultra-Violet Irradiation

Alkaline solutions of tyrosine and histidine become colored when exposed to ultra-violet radiation. CO_2 and NH_3 are evolved under the same influence. Tyrosine is probably the mother substance of the ultra-violet pigment. Histidine may be the mother substance of the irritants which cause erythema (83). Histidine yields ammonia on irradiation. This ammonia is formed from the nitrogen of the nucleus. The same reaction is obtained with proline, although somewhat less pronounced. Solutions of 0.5 per cent globulin and also 0.5 per cent casein show an evolution of ammonia if subjected to ultra-violet rays. Free amino groups are newly formed under this influence. This is not the case with 1 per cent casein. Tyrosine remains unchanged under these conditions while tryptophane is attacked. In the case of M/200 dipeptide solutions, neither evolution of ammonia nor new formation of amino groups could be observed. A peptone solution forms only a skin at the surface of the liquid if exposed to radiation.

These facts lead to the conclusion that the evolution of ammonia from casein under the influence of ultra-violet rays is dependent upon histidine groups and not peptide groups in the casein molecule. Gelatine, containing only 1 per cent of histidine, develops, however, much ammonia. This is due to the high percentage of proline and oxyproline in gelatine. Peptone does not form ammonia in spite of the presence of histidine, because of skin formation at the surface of the liquid. Arginine forms ammonia, but guanidine does not (84).

Mulzer & Schmalfuss have isolated 5.5 mg. of tyrosine from 2 kg. of skin. They assume that this amino acid is the mother substance of melanine (85).

Raper and others have investigated the behavior of N-methyl-dihydroxy-phenylalanine and N-methyl-tyrosine when acted upon by tyrosinase (86).

Blood Sugar

Synthetically prepared glycine has been found to lower the sugar content of blood. The same result is obtained in diabetics. Trimethylamine depresses the blood sugar, but the ethyl ester of glycine causes hyperglycemia. Glycine anhydride, glycocyamine, glycyl-glycine, glycyl-leucine, and methylamine are inactive (87).

The hyperglycemic effect of peptone is greater in rabbits which have been adrenalectomized than in rabbits which were splanchnicotomized. In dogs the reverse is true. Peptone and adrenaline with peptone produce in the adrenalectomized dog the same maximum blood-sugar value (88).

In phlorhizin diabetes, isoserin is eliminated as extra sugar, but β-hydroxy-γ-butyric acid is not glucogenetic (89).

Da-Rin and Repetti investigated protein metabolism and renal function in diabetes mellitus (90).

Action of Amino Acids in Anemia

Tryptophane and histidine are active in most cases of anemia, but their action is not always favorable (91). Kynurenin is inactive (92). Glutamic acid alone is ineffective in milk anemia, but a combination with Fe and Cu is effective (93). The glutathione content of liver preparations used in the treatment of pernicious anemia has been studied (94).

The hemoglobin content and the erythrocyte number of the salt frog are increased by hemoglobin, hematin, hematoporphyrin, pyrrole, and the iron compounds of pyrrole and citric acid (95).

Action of Amino Acids upon Growth

Phenylalanine, tyrosine, and tryptophane promote the growth of tadpoles. Glycine, arginine, and histidine are ineffective. Alanine occupies an intermediate position. The following amino acids, arranged in order of their effectiveness, promote metamorphosis: alanine, phenylalanine, tyrosine, tryptophane, iodotyrosine, and thyroxine (96).

The excretion of nitrogen by young pigs on a protein-free diet is lowered by asparagine, acetamide, and succinamide because of their sparing action on protein catabolism in the animal organism (97).

The absence of proline, oxyproline, glutamic acid, and hydroxyglutamic acid from the food of rats is without effect upon their growth (98).

The growth of larvae and eggs of fresh-water polyps and crepidulae is accelerated by the addition of cystine and cysteine. Partially oxidized cysteine has an inhibitory influence (99).

Toxic Action

In rats which have been maintained upon a basal diet of casein the weight of the kidneys increases after administering casein, glutamic acid, and glycine. The maximum effect is obtained after 10 days (100). Cystine causes the same effect but is inhibited by yeast extract (101). Disproportionate protein feeding causes an alteration in the liver (102). In rats which were fed with 4 per cent casein as nitrogen source, glycine, lysine, tryptophane, glutamic acid, and glutathione caused a more or less severe change in the liver, kidneys, and lens of the eyes (103). Peritoneal injection of amino acids (0.05 gm. in 1 per cent solution) causes nephrosis. The actions of cystine and glutamic acid compensate each other so that if both are injected no nephrosis results (104).

In animal nutrition, tryptophane (105) and histidine (106) are found to have minimum values and a maximum value as dietary constituents. If more than the nutritive maximum of histidine is used it acts injuriously, especially so in conjunction with glycine and alanine.

The administration of amino acids to the rats maintained upon a complete diet always causes disturbances. These disturbances are especially pronounced with 10 per cent cystine together with 10 per cent tyrosine, but less evident with glutamic acid. Ten per cent tyrosine together with 5 per cent cystine is less detrimental than 10 per cent tyrosine alone. It seems, therefore, that cystine lessens the toxicity of tyrosine (107).

Fermentation

Sake yeast in a modified Hayduck's solution produces fusel oil from the following amino acids added to the solution: glycine, *d*-alanine, *dl*-α-amino-*n*-butyric acid, *l*-leucine, *dl*-leucine, *dl*-valine, and *dl*-*n*-valine. Glutamic acid and aspartic acid form only traces of fusel oil. The fusel oil which results from aspartic acid consists of isobutyl- and isoamyl alcohol. The fusel oil from *dl*-α-amino-butyric acid is active amyl alcohol (108). The difference in fermentability of various kinds of molasses is conditioned by the protein content. After precipitation of the proteins, the molasses ferments more slowly depending upon the melanine content (109).

Serum Proteins

From the amino-acid content it appears that the chemical constitution of the human serum proteins shows variations from individual to individual (110). The serum-protein precipitates obtained by the use of vanadic, molybdic, and tungstic acid vary with the pH and the protein content of the various fractions (111). If fibrinogen is denatured by means of acids at 12–14° C. and pH 2.5–3.5, one finds the amino nitrogen value to be 8 per cent lower than otherwise (112).

The carbohydrate molecule in serum protein consists of mannose or galactose or both. This was found in the colorimetric analysis of protein by means of the orcine reaction. The albumin of horse serum contains equal quantities of mannose and galactose (113).

Urine Protein

Kozawa and collaborators have determined the following characteristics of isolated urine protein and of fresh and dialyzed urine: isoelectric point, pH 4.4; hydrolysis yields 80–82 per cent amino acids: 1.04–1.29 per cent histidine, 0.86–1.12 per cent tryptophane, 4.56–4.98 per cent tyrosine; albumin/globulin ratio, 1/1 (114).

These characteristics are in agreement with those of serum protein. Urine protein comes, therefore, from the serum. The correct value for the albumin/globulin ratio of urine protein is only obtained after dialysis.

Lang determined tyrosine, tryptophane, cystine, and arginine in urine protein and claims that urine protein originates from serum protein. Kidney protein has a somewhat lower cystine content than serum protein and the arginine content of its globulin fraction is higher compared with serum protein (115).

In the same individual it is found by ultrafiltration that the albumin/globulin ratios of urine and edema fluid are higher than that of serum (116).

In the detection of urine protein, acetic acid and 1 per cent flavianic acid produce a yellow precipitate. The precipitate which is formed by the action of quinine upon albumoses goes into solution on heating (117). Suyeyoshi's reagent is best suited for the clinical determination of urine protein; Esbach's method is of little value (118).

In many cases of melanosarcoma, melanogen occurs in the urine. The results which are obtained in the Thormählen test vary. This is an indication that dihydroxy-phenylalanine which is formed as an intermediary compound undergoes in pathological cases two types of degradation. One type involves the formation of pyrocatechol derivatives, soluble in ether, such as pyrocatechol-acetic acid. The other proceeds through indole derivatives, insoluble in ether, such as dihydroxy-indole-carboxylic acid, and involves ring closure (119).

Histidine may be determined quantitatively by Knoop's bromine test. N-methyl histidine and carnosine react negatively in this test while Im-methyl-histidine and histamine give a positive reaction with a different color. The histidine content of urine during pregnancy is 6–74 mg. per cent corresponding to 800 mg. of histidine per day and depends on the month of pregnancy (120).

In a case of cystinuria, lysine was isolated as the hydantoin in a quantity of 6.1 gr. in 60 liters of urine (121).

Ergothioneine is found to occur in normal urine (122).

The kidneys eliminate sulphates less readily than ammonia and urea (123).

The urine of dogs contains γ-butyrobetaine (124). Phosphorous poisoning causes γ-amino-β-hydroxy-butyric acid to be eliminated as unchanged hydroxy acid together with γ-butyrobetaine (125).

The ninhydrin reaction in human urine becomes more positive after physical exertion (126).

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MINERAL METABOLISM—SODIUM, POTASSIUM, AND CHLORINE*

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Total Minerals

Whereas the alkaline earths (and phosphorus) form the mineral framework of the supporting structures of the body, the monovalent bases (and chlorine) comprise the main components of the body fluids, and constitute the *milieu intérieur*. The mineral composition of the body organs has been described in detail (Heubner). The monovalent ions serve largely to maintain the osmotic and acid-base equilibria (Lucké & McCutcheon). Their relation to water metabolism is intimate, and hence to problems of blood and tissue hydration and excretion by the kidney, skin, and intestine.

But the effects of the electrolytes cannot be expressed by the osmotic pressure alone. They are not interchangeable anatomically or physiologically, as has long been known. The sodium is found largely in the intercellular body fluids, and the potassium in the intracellular fluids. The nature of the restraining force attributed to a "cell membrane" is still unknown.

Ion effect.—Ringer, Howell, and Loeb have shown that solutions containing sodium alone cause a relaxation of muscle, but when it is absent, contractility and irritability disappear. Potassium in the environment increases the irritability of tissues. The monovalent base effects are counteracted by those of the alkaline earths. Thus a physiological balance of sodium, potassium, calcium, and magnesium is essential.

Total base.—Any discussion of mineral metabolism considered from the point of view of the individual elements must, therefore, be not only fragmentary but often misleading, for it is not only the minerals themselves and their relation to one another but their summation also that is important in animal or plant economy. Such a point of view is attainable by a consideration of the "total base" in blood, which is now ascertainable by the simple method of Fiske. In addition, osmotic and acid-base effects may thus be examined. See the excellent reviews by Peters & Van Slyke[1] and Klinke.[2] This literature is not here reviewed.

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[1] See chapters xv and xviii.

[2] See especially chapter vii.

Acidosis.—The general problems of acidosis and alkalosis which involve not only the minerals but also CO_2, NH_3, Cl, and protein, and the physiology of the blood and respiration have been summarized by Peters & Van Slyke (cf. also Graham & Morris). The effects on urinary and fecal excretion and mineral balances comprise a more restricted field.

Acid-base metabolism.—The relations in metabolism, not only of individual elements, such as Ca/P or K/Ca, but of all the acids to all the bases, can be evaluated by the method of Sherman & Gettler, and of Berg (Salter, Fulton & Angier; Steffin). The algebraic sum of the two in relation to food intake, excretion in urine and feces, and retention, determines the acid-base value of metabolism.

Acids in food—those administered orally and parenterally, and those developed in endogenous metabolism—lead to elimination of bases: alkalies to excretion of acid radicals (Albright, Bauer & Aub; Lintzel; Wiley, Wiley & Waller). In diarrhea large amounts of sodium, potassium, and chlorine are lost. The relation of the "alkaline tide" of the urine to gastric secretion comprises a special problem in this field (Brunton).

Levels of intake.—Even when the mineral intake is optimal in regard to the acid-base and other relations as in a "complete" salt mixture, the total amounts may be too high or too low, and nutritional failure may result (Brooke & Smith; Lottrup). In dietaries of humans, as in those of farm animals (Hamilton), sodium chloride is required and universally employed (Orr).

Mineral metabolism.—Mineral metabolism studies during the growth of the infant fed with human and cow's milk (Swanson) yield an insight into the relations of intake to retention and to the composition of growth. Similar investigations for children and adults are rare, but increasing in frequency. Reports are available concerning the child on various normal diets (Hunscher, Cope, Noll & Macy), and on ketogenic diets (Weymuller & Ratner). The mineral balances have been followed during diurnal variation (Manchester), in dehydration (Wiley & Wiley; Hoffman & Post), in edema and diuresis (Bassett, Elden & McCann), in the acidosis of diabetes (Atchley, Loeb, Richards, Benedict & Driscoll; Peters, Kydd, Eisenman & Hald), in adrenal insufficiency (Loeb, Atchley, Benedict & Leland; Harrop, Soffer, Ellsworth & Trescher), and in *osteitis deformans* (Rabinowitch). The involution of simple goiter has been followed in rabbits (Baumann, Kurland & Metzger). The effects of

various light conditions, vitamin D, reduced pressure, and fasting on the sodium, potassium, chlorine, and also calcium and magnesium content of the skin have been demonstrated in the rat and guinea pig (Loewy & Cronheim).

Sodium

General summaries are found in Heubner, in Klinke,[3] in Meyer-Bisch, and in Peters & Van Slyke.[4]

Anatomy.—Cartilage, which is especially rich in sodium, has been analyzed for the other minerals also (Silber). Important new additions to the understanding of body composition have been rare. The difficulty lies in part in obtaining fat-free and blood-free tissues. The sodium of the blood serum comprises about 93 per cent of the bases, and in the blood cells of man it occurs in only small amounts, if at all. (In other species it may equal potassium.) In muscle most of the base is potassium. Presumably other organs are constructed on the same plan, the sodium occurring mainly in intercellular fluid, rather than in cells; therefore small contaminations with blood or variations in intercellular fluid cause large errors. The nature of the body water has been critically discussed (Peters & Lavietes).

The relation of sodium to muscular contraction in frogs has been studied (Mond & Netter). The long-continued ingestion of variable quantities of sodium is not without its influence on the tissues (Harpuder; Hermannsdorfer; Schaaf & Ormondt). Infra-red rays have been shown to increase the sodium of the blood (Malczynski). Subcutaneous NaCl is said to prevent development of rabbit carcinomata (Collier & Cohn).

The relation of the sodium and chlorine of cerebrospinal fluid (and other body fluids) has been reported as not inconsistent with the Donnan equilibrium theory (Dailey; Fremont-Smith & Dailey): contra (Gilligan, Volk & Blumgart).

Metabolism.—Normally the main source of sodium (and chlorine) lies in the salt used in cooking and at the table. That in the food is secondary. The sodium salts are easily absorbed, circulate readily, and are excreted principally by the kidneys, normally 90–95 per cent. More sodium, chlorine, potassium, and water are excreted during the daytime than at night (Manchester). Rats on low-salt diets excrete only traces of base (and chlorine) in the urine (Brooke & Smith). In normal men, according to the dictum of Bunge, excess sodium

[3] See chapter ix. [4] See chapter xv.

was found to cause excretion of potassium and vice versa (Wiley, Wiley & Waller) : contra (Bassett, Elden & McCann).

Sweat.—All forms of vigorous exercise or extreme dry heat lead to loss of NaCl in perspiration, and may result in "heat cramps." The concentration of NaCl in sweat is about 15 mM. per liter. Not more than 15 gm. of NaCl are lost daily (Dill, Jones, Edwards & Oberg). Replacement (in drinking water) leads to relief (Borchardt; Cassinis; Cassinis & Adilardi; Marchionini & Ottenstein; McCord & Ferenbaugh; Talbott & Michelsen; Whitehouse). The minerals of sweat have been determined (Talbert, Haugen, Carpenter & Bryant).

Edema.—Excess NaCl may lead to water retention in the normal adult. The infant is more susceptible, and the premature infant responds as an edematous nephritic [Paffrath & Massart (1 and 2)]. Unlike calcium, NaCl retention requires its quota of body water. In epilepsy (Proescher & Thomas) and pneumonia (Sunderman; Greenwald) it may be stored dry. Acidosis may be produced in infants [Fasold (1 and 2)] or adults (Raadt). When no NaCl but only dextrose was given to nephrectomized dogs no edema was produced, and life was prolonged fifteenfold (Barry, Shafton & Ivy).

Plasmapheresis or low-protein intakes without excessive salt lead to edema in experimental animals, similar to low-protein edema and war edema in humans (Weech & Goettsch; Weech & Ling; Weech, Snelling & Goettsch; Shelburne & Egloff). The effect on the intercellular spaces accumulates slowly as the plasma albumin drops. Excessive salt causes retention of sodium and leads to edema at a plasma-albumin level of 1.1–1.3 per cent; but at 1.7 per cent the dog responds as a normal. Similar findings were made from balance studies in nephrotic edema (Hoffman & Post).

Diuresis.—A high-salt intake apparently excites the kidney to excrete excess urine, and the diuresis thus produced may lead to negative sodium balances in the dog.

Studies of the mechanism of salt and urea diuresis show that the urine volume is not the resultant of additive effects as it is with the various cations (Gamble, McKhann & Butler; Farkas). With high-salt diets water is consumed in proportion to the amount needed for the production of urine. With saline drinking water the upper limit is reached at 1.5–1.7 per cent, and animals die of thirst rather than consume such solutions (Heller). Fasting, and acid-producing salts cause loss of sodium and potassium. So, too, do other diuretics (Blumgart, Gilligan, Levy & Brown).

NaCl is excreted in the urine when nephritic edema is reduced by diureses (Barker; Bassett, Elden & McCann; Thompson, Ziegler & McQuarrie). In nephritis the sodium, potassium, and chlorine excretion are diminished; potassium in the urine may exceed sodium, with either acid or alkaline diets [Glatzel (1, 2, and 3)]. The dissipation of edema in heart disease has been studied [Herrmann, Schwab & Bondurant; Scholtz (4)]. NaCl is lost in the diuresis of epileptics (Byrom; McQuarrie, Manchester & Husted) even with pituitary antidiuresis. The mechanism of parathormone diuresis has been investigated (Bassett, Elden & McCann; Thompson, Ziegler & McQuarrie).

Adrenal cortex.—The earlier work on adrenal insufficiency, produced by adrenalectomy, of Baumann & Kurland, and Hastings & Compere, has been confirmed for the dog, cat, rabbit, and man. An outstanding recent contribution is the discovery of the control of sodium (and water) metabolism by the adrenal cortical hormone [Loeb (1 and 2); Loeb, Atchley, Benedict & Leland; Harrop, Soffer, Ellsworth & Trescher]. Insufficiency is evidenced by low blood pressure and temperature, hemoconcentration, and loss of weight. The serum content of sodium (and chlorine) is lessened, and of potassium increased, and the NaCl and associated body water are excreted in the urine. The increased urea concentration has been described as a compensatory mechanism to maintain osmotic pressure. Administration of the active principle of adrenal cortex to men and dogs and administration of NaCl lead toward normalcy.

Potassium

General reviews of potassium have been recently given [Klinke;[5] Meyer-Bisch; Peters & Van Slyke[6]].

Anatomy.—The uneven distribution of potassium and sodium, the latter predominantly in the body fluids, and the former in the cells, remains an unsolved mystery. Little information has accumulated concerning the composition of tissues other than muscles and body fluids. The epidermis and cutis of guinea pigs yielded different values for total and ionized fractions of potassium (Bohnstedt). Values for rabbits' skin have been given (Steffin); a diminution was caused by X-rays (Adler & Wiederhold). It has been claimed that excess potassium intake caused a gangrene of rats' tails

[5] Chapter ix. [6] Chapter xv.

(Rost): contra (Duerst, Hugenin & Fischer). The potassium content of tumors was higher than that of other tissue, and KCl increased their growth (Epstein): contra for chicken sarcoma (Morávek). Values both higher and lower than normal tissue were found in uterine tumors of various stages of development [Scott (2)]. The potassium content of bone marrow in cases of carcinoma has been investigated (Hoffmann). The rôle of potassium (also of sodium, calcium, and magnesium) in cancer has been exhaustively reviewed (Shear).

Muscle.—The nature of the combination and the function of potassium in muscle are not understood. The finding of Neuschloss of "bound potassium" in rabbits' muscle was not confirmed (Callison). The potassium is said to be present in indiffusible form, and to be liberated during contraction (Ernst & Takács). The potassium content of red and white muscles, and of heart and stomach, varied over 100 per cent even in different pieces from the same muscle (Steffin; Millard).

Comparisons of potassium in normal and pathologic heart muscle have yielded conflicting data [Harrison, Pilcher & Ewing; Leulier, Pommé & Delaye; Leulier, Pommé & Richard; Scott (1); Wilkins & Cullen]. The potassium content of the heart, skeletal muscle, liver, and kidneys of men and dogs was lowered by work and not by edema, and was restored by potassium administration [Calhoun, Cullen, Clarke & Harrison (1 and 2); Calhoun, Cullen & Harrison]. Long-continued administration of KCl caused an increase of potassium in the spleen, kidney, and perhaps the heart of rats (Harpuder). After digitalis the potassium content of the heart, but not of other muscles, of dogs was reduced (Calhoun & Harrison). Thyroid intoxication was without effect on the potassium (and sodium) of heart muscle (MacKay & Bergman). Section of the sciatic nerve was accompanied by increase in chronaxia and fall in potassium when the muscle degenerated (Leulier, Pommé & Richard). Paralyzed muscles also showed a diminution (Leulier, Pommé & Bernard). The potassium content of bile, milk, and colostrum have been reported (Steffin).

Blood.—The distribution of potassium between cells and serum differs according to species. Human blood contains about 20 mg. per 100 cc. in the serum, and 420 mg. in the cells. The more rapidly plasma was separated from cells, the less potassium it contained (Dulière). The thesis that serum potassium is in part colloidal was

deduced from ultrafiltration experiments [Scholtz (1 and 2)]. Serum potassium showed slight variations under any conditions (Kotikoff; Lévy & Pacu).

Because of blood findings, intracardiac KCl followed by $CaCl_2$ has been recommended in electric shock (Petrov & Libikh). Malnutrition retarded the return to normal after fatigue (Schlutz, Hastings & Morse). In idiopathic tetany, sodium and potassium were reported as normal [Scholtz (3)], or the potassium increased (Unshelm). Manic depressive insanity was found to be associated with electrolyte instability—a tendency to increased potassium, calcium, and pH, and diminished sodium [Tómasson (1 and 2)].

Serum contains more potassium than the cerebrospinal fluid (Dulière; Massaut) or pleural effusions (Dumitresco-Mante & Petrovano). The potassium in cerebrospinal fluid was increased after hemorrhage (Ballif & Gherscovici). KCl injection caused a rise in the potassium content of both serum and aqueous humor (Räihä).

Metabolism.—Because potassium salts cannot be stored in body fluids but only in cells, they have long enjoyed an important place as diuretics. Administration to two normal men and to an edematous nephritic resulted in no alteration of the sodium balance in the former, but in the latter caused NaCl excretion along with water loss (Barker; Bassett, Elden & McCann). Water storage in epileptic children was associated with potassium excretion (McQuarrie, Manchester & Husted).

Acidosis and acid-producing salts, as shown by Gamble, lead to the excretion of body water and tissue destruction; if the fluid is extracellular, sodium is lost from the body; if intracellular, potassium is lost. The potassium, in excess of intake, found in the urine has been used as a measure of tissue-water elimination, with or without protein destruction, as in the studies of diuretics (Blumgart, Gilligan, Levy & Brown), of diabetic acidosis (Peters, Kydd, Eisenman & Hald; Atchley, Loeb, Richards, Benedict & Driscoll), dehydration (Wiley & Wiley), ingestion of NH_4Cl and $NaHCO_3$ (Wiley, Wiley & Waller), ketogenic diets (Byrom), and in nephrotic edema (Hoffman & Post).

The potassium in sweat must be taken into account in mineral balances (Atchley, Loeb, Richards, Benedict & Driscoll; Swanson; Swanson & Iob). In experimental scurvy sodium and potassium elimination were decreased, and serum sodium increased [Michaux (2); Randoin and Michaux (2)].

K/Ca.—The literature on K/Ca has been recently reviewed (Shohl). Because potassium is known to increase irritability and calcium to diminish it, interest in this relationship continues. In addition to the previous studies are found those of the effect on absorption from the intestine (Gellhorn & Skupa), the relation of ultrafiltration in kidney insufficiency [Scholtz (2)], and thyroparathyroidectomy on the tissue content (Underhill & Jaleski).

Na/K.—The problem of Na/K which was first emphasized by Bunge and later by Ringer, Loeb, and Howell has again become active. Baumann & Kurland, and recently Harrop have suggested that the state of the circulation in adrenal insufficiency may depend upon this relationship: the Na/K in serum may fall from over 20 to less than 10. Similar data are given for cardiac and striated muscle, kidney, and liver (Cullen, Wilkins & Harrison). If the sodium represents intercellular and the potassium intracellular fluid, the relationship is properly discussed in milli-equivalents per kilo of water (Wilkins & Cullen). So expressed, Na + K should represent the amount of fluid in tissue and the ratio, Na/K, the relation of intercellular to cellular fluid.

Good fodder grass has an Na/K of $\frac{1}{18}$, and is alkaline; additions of NaCl and acid grains balance the diet (Lintzel). When the Na/K of food of experimental animals, normally $\frac{1}{6}$, was changed to $\frac{1}{542}$, hyperirritability was present. The effects of alterations in Ca/P, K/Ca, and Ca/Mg were also important in the production of tetany (Seekles & Sjollema). Diminution of Na/K in intake has been found beneficial in edema of humans (Barker), though, in nephritis, potassium must be used with caution. The potassium of gastric juice was found to be greater than that of serum, and the sodium much less (Ingraham & Visscher). The Na/K of sweat is normally 10 (Talbert, Haugen, Carpenter & Bryant).

Chlorine

Little has been written in the past year that changes the information summarized by Peters & Van Slyke,[7] Klinke,[8] Heubner, and Meyer-Bisch. The question of the form of chlorine is under examination and the proof of organic combinations would be welcome, but convincing evidence is not at hand [Peters & Van Slyke;[9] Sunder-

[7] See chapter xix.

[8] See chapter ix.

[9] Footnote on p. 1019.

man & P. Williams (1 and 2); Hastings, Harkins & Liu; Morris, Watson & Morris].

Anatomy.—That the skin and subcutaneous tissue may serve as a depot for chlorine is well established—in infancy more than in the adult (Török).[10] This chlorine is not replaceable by administered iodine [Shoemaker & Underhill (1 and 2)], even under conditions of need for chlorine, as in the edema caused by superficial burns. The ability of the skin to absorb NaCl, serum, and colloids is affected by the NaCl intake (Adlersberg & Perutz).

The chlorine contents of muscles, liver, and kidneys in experimental scurvy have been given [Randoin & Michaux (1)].

Blood.—Chlorine is intimately connected with sodium in osmotic relationships, and hence in water metabolism. In blood, chlorine passes across the "cell membrane," whereas sodium and potassium cannot, and therefore is important in acid-base relationships and the Donnan equilibrium. It forms about two-thirds of the acids of the blood serum. Taken together with $NaHCO_3$, with which it varies inversely, it maintains the stability of the blood acids and osmotic pressure.

The cells contain only about half as much chlorine as plasma. Studies have been made of the distribution of chlorine (and bromine) in relation to the Donnan membrane equilibrium (Hastings & Van Dyke; Van Dyke & Hastings), in various diseases (Muntwyler, Rose & Myers), in shock (Coehlo & Rocheta), and after irradiation with ultra-violet light (Glass). Diminution of chlorine has been reported in pneumonia (Greenwald; Sunderman), in tuberculosis (Gatta), in malaria (Manca), and in dehydration of infancy [Kerpel-Fronius (1 and 2)], and an increase has been observed in scurvy [Michaux (1)] and after administration of splenic extracts (Schliephake & Probst). Ingestion of glucose leads to a diminution of serum chlorides (Sunderman & Williams). The effect of NaCl in reducing blood sugar of diabetics reported by Peters has been confirmed (Glass & Beiless). Its further beneficial effects on blood electrolytes in diabetic acidosis have been detailed (Kydd). The chlorides in the blood in nephritis and other pathological conditions have been reviewed (Peters & Van Slyke, chapter xix). When hypochloremia exists, in the absence of cardiac or nephritic edema, chlorides may be used effectively (Kalapos). The low serum chloride in adrenal

[10] Cf. also p. 212.

insufficiency has been discussed in the section on adrenal cortex under "Sodium."

Cerebrospinal fluid.—The chlorine in cerebrospinal fluid (Stahl, Weill, Deviller & Grabar), lymph (Heim) and amniotic fluid (Shrewsbury) or in ultrafiltrates exceeds that in serum. The chlorine distribution between serum and cerebrospinal fluid has been studied in relation to the Donnan equilibrium (Coombs & Thompson; Fremont-Smith & Dailey; Fremont-Smith, Dailey, Merritt, Carroll & Thomas; Muntwyler, Way & Pomerene; Rothschild & Malamud; Gilligan, Volk & Blumgart). Similar relations in nephritis and NH_4Cl acidosis were found (Linder & Carmichael).

Gastric secretion.—Gastric acidity is not influenced by sodium chloride intake, but is affected by the composition of the blood (Gilman & Cowgill): contra (Sunderman). The effect of gastric secretion on arterial and venous blood has been described (Kurchakow). Under histamine stimulation the secreted gastric juice has been shown to be of constant chlorine content and acidity. The variations usually found are attributed to mucus and base (Austin & Gammon; Hollander). With vomiting, as in high intestinal obstruction or pyloric stenosis (Morris & Graham) chlorine is lost from the body, and blood- and tissue-chlorine diminished (Maizels; Schnohr). This, with the concomitant alkalosis, has been described as the cause of tetany. The same cause has recently been given for the convulsions in water intoxication (Smyth, Deamer & Phatak). Changes in the osmotic pressure in the tissues are also present (Gömöri & Molnar).

Achlorhydria has been critically reviewed (Oliver & Wilkinson).

Metabolism.—The interrelationship of sodium and chlorine is so close that it is often impossible to separate their effects, and many problems are best reviewed as those of NaCl metabolism.

About 10–15 gm. of sodium chloride constitute an average daily consumption. Diminished intake leads to a practical cessation of excretion; and high intake of NaCl, even in normals, may lead to retention or edema. Low intake with oat diets causes in rabbits a fall of serum sodium, chlorine, and potassium to between one-half and one-third normal, with death in convulsions in ten days; similar blood findings occurred in dogs (Lottrup). Ingestion (Weech & Goettsch) and injection (Dresel & Leitner; Smirk) of hypertonic salt solutions in relation to excretion have been investigated. The relation of chloride balances to surgical operations has been studied (Stewart, Talbott & Churchill).

Chlorine is easily absorbed into the blood and is excreted normally, almost wholly in the urine. Glomerular urine chloride represents an ultrafiltrate of plasma (Westfall & Richards). The laws governing excretion are not as simple as Ambard once supposed, for Peters and others have shown that chlorine may be excreted when the serum chloride is below the "threshold value" (Hastings, Harkins & Liu; Möller). The relations of the anterior and posterior hypophysis to diuresis are especially important, and have been reviewed (McQuarrie, Manchester & Husted). The amounts of chlorine in feces and sweat show variations, but are usually small compared to those in urine (Mosher). In diarrhea great losses of chlorine may occur by the bowel. In profuse sweating, the chlorine thus lost may exceed that in the urine (Dill, Jones, Edwards & Oberg). Cf. also p. 212.

Such a review is by necessity fragmentary, for many important phases of the subject, e.g., the mineral composition of the body organs, may be investigated infrequently. Science may be said to increase *per saltum* rather than *per annum.*

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MIRROR LAKE
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THE HORMONES*

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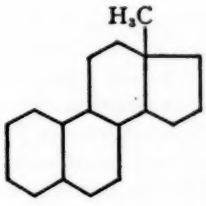
Oestrin.—Adam *et al.* have indicated the proper nomenclature for substances of this group, by reference to the fundamental saturated hydrocarbon, oestrane, $C_{18}H_{30}$, which is 2- (or possibly 1-) methyl-1,2-cyclopentano-perhydrophenanthrene (Formula I), and have suggested the attractive terms "oestrone" for ketohydroxyoestrin (theelin, menformon) and "oestriol" for trihydroxyoestrin (theelol). If the sterol system of numbers is applied, the latter may be called 3,16,17-trihydroxy-1,3,5-oestratriene, accepting the tentative formula mentioned in last year's review. The dibasic acid obtained therefrom by fusion with potash (Formula II) was found by Butenandt, Weidlich & Thompson to lose two molecules of CO_2



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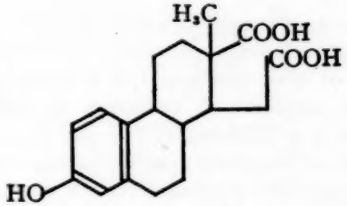

II

during dehydrogenation with selenium, yielding a dimethyl-phenanthrol, which after reduction with zinc gave 1,2-dimethyl-phenanthrene (Formula III). This substance, previously unknown, was also obtained from aetiobilianic acid, and was also prepared synthetically by selenium dehydrogenation of the product obtained by the action of a Grignard reagent on the methyl-cyclohexanono-naphthalene (Formula IV) obtained by condensing naphthalene with methyl-succinic anhydride. This appears to settle the formula of oestrane (Formula I) except that the methyl group might be at C_{14} rather than C_{13}. For each of these alternatives there are two possible positions for the alcoholic hydroxyl groups of oestriol, since if *gem*-dimethyl groups were formed in the formation of the dimethyl-

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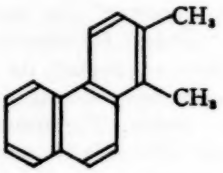
phenanthrol, migration would probably take place. Moreover, the position of the phenolic hydroxyl is quite uncertain, though Schwenk & Hildebrandt (1) point out that the similarity of certain colour reactions with those of *ar*-tetrahydro-β-naphthol suggests that C_3 is the most probable location; this accords also with the surface-film properties (Danielli, Marrian & Haslewood). MacCorquodale, Levin, Thayer & Doisy continued the oxidation of the methyl ether of



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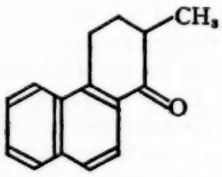


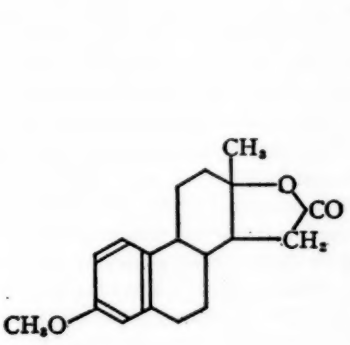
IV

the acid (Formula II) and obtained a lactone (Formula V) and other derivatives of uncertain constitution. It is very interesting that most of their products had a greater biological activity than the native hormone. A reduction product of oestrone, obtained by Schwenk & Hildebrandt (2) and Girard, Sandulesco & Fridenson, was also more active than the parent substance. Sandulesco, Tchung & Girard have published further details on the naturally occurring equilenin, an oestrapentaene probably containing a naphthalene nucleus.

Cook & Dodds obtained oestrogenic reactions with synthetic 1-keto-1,2,3,4-tetrahydrophenanthrene (Formula IV *minus* the methyl group), 9,10-dihydroxy-9,10-dibutyl-9,10-dihydro-1,2,5,6-dibenzanthracene (Formula VI), 5,6-cyclopenteno-1,2-benzanthracene, and 1,2-benzpyrene. It is very interesting that the two last are also powerfully carcinogenic. Ergosterol, neo-ergosterol, and calciferol (vitamin D) also possessed some oestrogenic activity, the first-named being the weakest. Cook, Dodds, Hewett & Lawson find that closely related substances may be inert.

The most active of these synthetic substances showed also the oestrin effect in plumage tests. It is well known, from the work of Juhn & Gustavson and Freud, de Jongh & Laqueur (confirmed recently by Zawadowsky and Mitchell), that in ordinary fowls and ducks the "henny" type of feathering depends solely upon the pres-

ence of oestrin, whether produced endogenously or supplied experimentally. On the other hand, in the Sebright fowl (Gallagher, Domm & Koch) and in the black-headed gull (Oordt & Junge), oestrin and testicular hormone have similar effects and it is only in castrates that the type of plumage usually regarded as male can appear. In the sparrow (Keck) and probably in the turkey and guinea-fowl, the plumage type is genetically determined and is not influenced by sex hormones.



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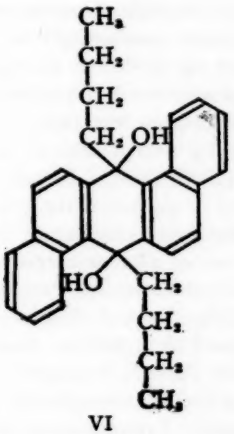

VI

The assay of oestrin continues to attract attention. The apparent potency depends on the medium in which injections are made, on the division of the dose, the type of vaginal smear regarded as a positive response, and, not least, the frequency with which the smears are examined and the times selected for examination. The use of oily mediums is condemned by Deanesly & Parkes, who show that the oil and some of its oestrin content may remain at the site of injection almost indefinitely. When an aqueous medium is used, the rat unit is little larger than the mouse unit (Burn & Elphick), while injections in oil are relatively far more effective in mice (Kreitmair; de Jongh & Laqueur), and the difference between oestrone and oestriol is also exaggerated (Burn & Elphick). Girard, Sandulesco & Fridenson also draw attention to the importance of the solubility of the substance tested, with special reference to oral administration.

The comparatively high activity of oestriol in immature intact rodents is also discussed by Browne, and Butenandt & Browne.

Laqueur, Freud & de Jongh challenge the usual view that oestrin has no direct effect on the male genital system, since they believe that it induces a development of the seminal vesicles of the castrate male rat; this is ascribed to muscular hypertrophy rather than to the epithelial development obtained with testicular hormone.

Anterior hypophysis.—The most striking advance in the period under review has been the clear demonstration of the presence of several separable hormones in this organ. Riddle, Bates & Dykshorn (1) show convincingly that their "prolactin" fraction, which is obtained by isoelectric precipitation and causes growth of the crop-gland in pigeons and secretion from the developed mammary glands of rodents, is free from the other known effects of crude pituitary extracts, except that it inhibits the development of the gonads (Riddle & Bates). Evans, Meyer & Simpson, in an elaborate study of the properties of the growth hormone, found it possible to get rid of the gonad-stimulating factor by extracting the acetone-insoluble fraction of alkaline extracts with glacial acetic acid; but their preparations still stimulated the thyroid and adrenals, which those obtained by Collip, Selye & Thomson (1) did not. These latter extracts were obtained by a process involving adsorption on calcium phosphate; Riddle, Bates & Dykshorn (1) found them to be free of gonad-stimulating and thyreotropic effects but to be somewhat contaminated with prolactin. From extracts from which the growth-hormone had thus been removed, Anderson & Collip prepared an active thyreotropic fraction. This however was not free from adrenotropic activity; Loeser indeed suggests that adrenal hypertrophy is an inevitable consequence of the hyperthyroidism produced, and such extracts should therefore be tested on thyroidectomized animals. Riddle, Bates & Dykshorn (2) draw attention to the fact that most thyreotropic extracts possess gonad-stimulating properties; but there is ample evidence, for instance in the work of Evans, Meyer & Simpson, to suggest that this association is accidental. The thyreotropic extracts of the German workers do not seem to have been critically examined for their physiological purity. Collip, Anderson & Thomson obtained a thermostable fraction which produced excellent repair of the adrenal cortex in hypophysectomized rats without affecting the thyroid, and which was free of growth and gonad-stimulating principles. It is clear that there is good evidence for the existence of

at least five separable active principles in the anterior hypophysis. The use of the term "growth hormone" as a synonym for "crude hypophyseal extract containing an assortment of hormones" is therefore to be deplored; and the complexity of the situation should be recalled when physiological interpretations of the histological appearance of normal or pathological hypophyses are under consideration.

Other effects of the injection of hypophyseal extracts do not yet require the predication of still more hormones. The diuretic action is ascribed by Barnes, Regan & Bueno to the thyroid over-activity. Similarly the "pancreatropic" action (increase in number and size of the islets) seen by Anselmino, Herold & Hoffmann in rats and by Aron in foetal guinea-pigs injected *in utero* is related by the latter to the similar action of thyroid hormone (Florentin & Watrin). Eitel, Löhr & Loeser similarly find that the increase in acetone-body production (Burn & Ling; Butts, Cutler & Deuel) is not obtainable after thyroidectomy. This was not true of Funk's preparations from normal urine.

Houssay's striking demonstration that hypophysectomy lessens the severity of pancreatic diabetes in dogs has been confirmed by Barnes & Regan, and by unpublished experiments elsewhere. The tendency of hypophysectomized rabbits to spontaneous hypoglycaemia (also noted in dogs by D'Amour & Keller, and Daggs & Eaton), and their sensitiveness to insulin and resistance to the hyperglycaemic action of adrenaline have been carefully studied by Corkill, Marks & White. There is reason to suppose that these effects do not depend upon the thyreotropic activity of the hypophysis; thus the tendency of crude extracts to produce, in time, diabetic symptoms in normal animals (Houssay; E. I. Evans) is not obtainable with thyreotropic concentrates (Lucke, Heydemann & Duensing), which, according to Horsters, tend indeed to lower the blood sugar. It has also been suggested that the influence of the hypophysis on carbohydrate metabolism is exerted through the adrenal cortex, rather than directly, but at present no evidence is available on this point.

It is, indeed, a matter of dispute how far symptoms of adrenal insufficiency appear after hypophysectomy. Baird, Cloney & Albright showed convincingly that the low resistance of hypophysectomized rats to cold could be improved by administration of cortical extracts; Atwell felt that this applied to many of the symptoms of cachexia in such animals, probably including the low blood pressure (Menendez), although Evans, Swingle, *et al.* found cortical extracts

of little value in comparison with the restorative action of "growth-hormone" preparations.

The effect of thyreotropic preparations on basal metabolic rate has been studied in guinea-pigs by Schoedel, Diefenbach, and Friedgood, and in rats also by Szarka, and Anderson & Collip. Two general rules that emerge are, first, that the maximum increase is much less than that obtainable by thyroid feeding and usually under 40 per cent, and secondly that there is a striking tendency to return to normal or sub-normal levels in spite of continued injection. The claim that the thyreotropic effect depends on the integrity of the cervical sympathetic is denied by Krayer; moreover, Houssay, Biasotti & Magdalena obtained a reaction from transplanted thyroids and Eitel, Krebs & Loeser actually produced hyperplasia in sliced thyroid tissue *in vitro*. Despite the observations of Hellwig, Nielsen, and others, it seems that the thyreotropic hormone is not excreted in the urine in Graves's disease or otherwise, and may indeed be incapable of passing the kidney (Krogh & Okkels; Smith & Moore) or the placenta (Aron).

The problem of the gonad-stimulating substances found in certain human urines has become somewhat clearer. Collip, Selye & Thomson (2) describe in some detail the action of pregnancy-urine preparations on the ovary of the hypophysectomized rat; there is no enlargement of follicles nor formation of corpora lutea, but the thecal cells (whose appearance is strikingly altered after hypophysectomy) are enlarged and luteinized. These observations have been confirmed by Wade, and are in agreement with those of Kraul and of Evans, Pencharz, Simpson & Meyer; and although Leonard & Smith (1) did observe the formation of corpora lutea, they agree that hypophysectomy entirely changes the ovarian response to such preparations. Selye, Collip & Thomson (1) extended their studies to hypophysectomized mice. Engle observed that the effects of pregnancy urine on the ovary of the macaque can hardly be described as stimulating, and the irresponsiveness of birds is, of course, well known. Normal rabbits respond well to these substances, but White & Leonard, Firor, and Hinsey & Markee (1) all find that they gradually become refractory after hypophysectomy.

We may emphasize the word "gradually" above, since it appears to rule out the suggestion that pregnancy urine contains, along with a small amount of gonad-stimulating substance, a relatively large amount of hypophysis-stimulating substance. Far more attractive is

the interpretation that pregnancy-urine principles can act fully only in the presence of a complementary or synergistic substance which is supplied by the hypophysis of the treated animal (rat, mouse, or rabbit). This is presumably to be identified with the substance in hypophyseal extracts which so greatly enhances the action of pregnancy-urine preparations in intact rodents, and which, according to Evans, Simpson & Austin, and Collip, Selye, Anderson & Thomson, cannot be completely separated from the hypophyseal gonad-stimulating substance.

That the gonad-stimulating substances of pregnancy urine are fundamentally different from those of hypophyseal extracts there can be no doubt, and we are glad to note an increasing tendency to suppose that the former are actually produced by the placenta. Nevertheless, the alternative hypothesis that the placenta merely collects and in some way modifies the hypophyseal hormone has little evidence against it; Philipp, however, described a case in which a hydatidiform mole was so thickly encapsulated with fibrin as to be almost cut off from the circulation; gonad-stimulating substances were abundantly present in the mole but not in the blood and urine.

It follows as a corollary to this view that when gonad-stimulating substances occur in the urine in the absence of tissue of placental type (so-called "prolan A without prolan B") they must be of different origin, presumably hypophyseal, and of different nature. Evans, Simpson, Austin & Ferguson showed that these substances resembled hypophyseal extracts and differed from placental or pregnancy-urine preparations in their power to produce almost unlimited hypertrophy of the ovary, and in being active (according to Riddle) in birds; they are also fully active, according to Leonard & Smith (2), in hypophysectomized rats. Hamburger finds that they differ from pregnancy urine in that the ratio of the rat unit to the mouse unit is as 1 to 2 instead of as 5 to 1.

The last support for the theory that pregnancy urine contains separate follicle-stimulating and luteinizing substances (prolan A and prolan B) of which only the former is present in other urines has therefore been withdrawn. The use of the term prolan is objectionable, not only on account of its association with this theory, but because of its commercial application, and because it has frequently been employed in describing hypophyseal extracts; on the other hand, it is a convenient word, and is fairly widely understood to mean "gonad-stimulating substance of pregnancy or chorion-epithe-

lioma urine." To others, the description of this substance as "anterior-pituitary-like" has commended itself. Most of the other terms in use obscure the distinction between urinary and hypophyseal preparations.

The gonad-stimulating substance of the serum of pregnant mares is exceedingly potent in both normal (Cole, Guilbert & Goss; Evans, Meyer & Simpson) and hypophysectomized rats (Evans, Pencharz, Simpson & Meyer), and is therefore hypophyseal in type and presumably in origin, and may indicate a function of the hypophysis in pregnancy which in man has been taken over by the placenta.

The problem of gonad-stimulating substances in the hypophysis itself is harder to solve. It is not impossible that active extracts contain (*a*) a substance physiologically similar to that found in pregnancy urine and (*b*) a substance, possibly inert in itself, which acts in a synergistic or complementary manner with the foregoing and with pregnancy-urine preparations. Thus when Collip, Selye & Thomson (2) found that very young female rats responded to pregnancy urine as hypophysectomized rats do, they concluded that at this early age the complementary substance was not yet being produced; and Swezy found that the hypophyses of such infantile rats behaved like pregnancy-urine preparations in being active in normal but not in hypophysectomized rats. Variations in response to pituitary treatment according to the age (Lipschütz) or species (Loeb) of the donor or according to the method of preparation (Fevold, Hisaw, Hellbaum & Hertz; Wallen-Lawrence) might then be ascribed to different ratios or partial separation of the two fractions, which must, however, be assumed to remain in company in their passage into the blood and urine. As yet the evidence hardly warrants serious statement of any such theory. It is by no means impossible that the "complementary" effect (in combinations with pregnancy urine) is merely one of the properties of the true hypophyseal gonad-stimulating substance, which in turn may be complex in nature, or may even owe its apparent complexity to the accompanying thyreotropic principle (Fluhmann; Loeb), which may alter the reaction of the gonads by the effect of thyroid hormone thereon (Schockaert; Van Horn; etc.).

Some progress has been made in the purification of the gonad-stimulating substance by Van Dyke & Wallen-Lawrence, and by Evans, Meyer & Simpson, who made use of ammoniacal acetone and alcohol. The serum of pregnant mares lends itself more readily to

purification, and Evans, Gustus & Simpson have used an adsorption technique successfully. Evans, Meyer & Simpson, Lejwa, and Zondek, Scheibler & Krabbe have all reported methods for the purification of pregnancy urine; but more attention has been given to the complete recovery of the gonad-stimulating substances therefrom, Davy & Sevringhaus and Elden using absorption, while Katzman & Doisy use tungstic-acid precipitation. Activity in normal urines may be demonstrated by these methods, or by the addition of hypophyseal extract to enhance the effect (Evans, Simpson & Austin). Haurowitz, Reiss & Balint draw attention to the presence of considerable amounts of carbohydrate and of arginine in purified pregnancy urine preparations.

Many workers, including Riddle, Bates & Dykshorn (1), Gardner & Turner, E. I. Evans, Gaebler, and Lyons & Catchpole, have induced lactation by administration of hypophyseal extracts; Lyons, Chaikoff & Reichert obtained this effect in hypophysectomized dogs. The view is widely held, for example by Turner & Frank, that development of the mammary glands occurs under the influence of oestrin and progestin in combination, and that the hypophyseal "prolactin" then induces actual secretion. This view may be too simple to be applied to all species; thus Nelson & Smelser obtained full mammary development in male guinea-pigs with oestrin alone, and Ruinen found that hypophysectomy did not prevent this action; and again, though Asdell & Salisbury reaffirm the importance of progestin in mammary development in the rabbit, Lyons & Catchpole maintain that prolactin causes actual growth. Pencharz & Long, and Collip, Selye & Thomson (2) find that rats may be hypophysectomized up to ten days before parturition without abortion occurring; such animals lactate for a few hours after parturition, which suggests that the pregnant uterus may be able to stimulate milk secretion when the hypophysis is absent; Selye, Collip & Thomson (1) confirmed these results in mice. Grauer & Robinson observed milk secretion in transplanted mammary tumours, which is further evidence that nervous stimulation plays no part.

What stimulates the hypophysis to secrete prolactin? It has been suggested (Nelson & Smelser; Smelser) that a sudden fall in the amount of ovarian hormone in circulation may act as a stimulus. This view is supported by Collip, Selye & Thomson (2), who found that the mammary glands of virgin rats were developed by the hormones liberated by ovaries under "prolan" stimulation, and came

into secretion when the ovaries were removed; moreover, lactation may be inhibited by oestrin (de Jongh; Smith & Smith) or by substances which stimulate oestrin secretion [Enzmann & Pincus; Selye, Collip & Thomson (2)]. This cannot be the only factor; Nelson found that Caesarian section, in the rat, did not lead to lactation if the placentae were not removed, but since Litt did not find placental transplants inhibitory in the rabbit, and Selye, Collip & Thomson (2) found complete Caesarian section ineffective in rats if the uterus was immediately distended with paraffin, this influence may be nervous rather than hormonal. Selye, Collip & Thomson (2) also found good evidence that the act of suckling reflexly maintains prolactin secretion (which must be continuous throughout lactation, since hypophysectomy at any point interrupts the secretion).

The nervous pathways involved in such reflexes are obscure; in fact, the whole question of innervation of the anterior hypophysis is difficult. If further proof were needed that copulation causes a discharge of hypophyseal hormones in the rabbit, it is supplied by the cross-circulation experiments of McPhail, Parkes & White; yet destruction of the cervical sympathetic does not prevent this response, according to Vogt and Hinsey & Markee (2) (cf. also Haterius). The rabbit type of mechanism of ovulation is found also in the ferret (Hill & Parkes; McPhail) and most probably in the cat, where again sympathectomy has no effect.

For the central position which the anterior hypophysis is rapidly assuming in the endocrine system, its manifold activity is hardly more important than its manifold responsiveness. That changes in the activity of the gonads may be reflected in histological and other changes in the anterior lobe is well known; though it is uncertain whether the changes parallel to the oestrus cycle (Charipper & Haterius; Wolfe & Cleveland; Wolfe, Cleveland & Campbell; Andersen; Andersen & Kennedy) are to be regarded as causes or effects. The changes in structure and function that may follow the administration of "prolan," as studied by Desclin, by Collip, Selye & Thomson (2), by Lucarelli, by Wolfe, Phelps & Cleveland, and by Leonard, are uniformly regarded as secondary to the changes in ovarian endocrine function; only Leiby, and possibly de Jongh, see evidence of a direct effect. Thyroidectomy also affects the structure of the anterior lobe (Hohlweg & Junkmann; Pugliese; Van Dyke & Ch'en), and if thyreotropic potency is not increased thereby (Houssay, Novelli & Sammartino) it may be decreased by thyroid admin-

istration (Kuschinsky). The influence of the adrenal cortex on reproductive activity seems hardly definite enough to be direct [Cleghorn; Loos & Rittmann; Corey & Britton (2)], and Martin found evidence that it depended on hypophyseal changes; this may apply also to the effect of adrenalectomy on lactation, which Brownell, Lockwood & Hartman regard as direct. Howard & Grollman agree that the cortex has no function in reproduction.

The relation of the hypophysis to tumour growth has received attention; Ball, Samuels & Simpson, McEuen & Thomson, and Reiss, Druckrey and Hochwald agree that hypophysectomy retards the growth of transplantable rat tumours, and Guyer & Claus saw morphological changes in the anterior lobe in tumour-bearing rats. Hamburger suggests that, while certain tumours produce "prolan" themselves, others incite the hypophysis to pour out gonad-stimulating hormones. The claim of Zondek, Zondek & Hartoch, and Möller, that "prolan" specifically inhibits tumour growth, is denied by Wiesner & Haddow and others.

Evocation of nest-building and other maternal behaviour patterns after injection of gonad-stimulating hormones has been noted in rats by Wiesner & Sheard, and in rabbits by Tietz.

Corpus luteum.—The hormone progestin (corporin) has been purified and crystallized by Fevold & Hisaw, and Allen; but its chemical nature is still unknown. Engelhart suggests that its destruction by pancreatic juice shows it to be an ester. The substance which renders the rabbit uterus insensitive to pituitrin is apparently distinct from this (Illingworth & Robson; Fremery, Luchs & Tausk). Other activities of the luteal tissue remain uncertain, since oestrin alone may produce relaxation of pubic ligaments (Brouha) or vaginal mucification (Meyer & Allen), yet it is not certain that the activity of luteal extracts in these respects is wholly due to their oestrin content. Allen & Meyer find that oestrin may be withdrawn from progestin dissolved in petroleum ether by washing with dilute alcohol.

Thyroid.—Rees & Salway observe that with modern methods of desiccation the proteins are not completely denatured, so that it is wrong to assume that all the iodine extractable with water is inorganic; Harington & Randall, and Lawson, therefore propose to complete the denaturation by using hot alcohol for extraction. Blau has modified the butyl-alcohol method for separating thyroxin from di-iodotyrosine.

Salter, Lerman & Means used the polypeptide obtained by enzymic hydrolysis of thyroid tissue to make a comparison of thyroxin (which is ineffective orally because of its insolubility) with crude desiccated thyroid. They found that by injection (in myxoedematous patients) the polypeptide was just as active as thyroxin, in doses of equal iodine content; and the activity of the polypeptide was almost the same orally as intravenously. Means, Lerman & Salter then found that desiccated thyroid was almost as active as thyroxin-polypeptide, in equal-iodine doses by mouth, although it was clearly shown that less than a quarter of the iodine of the former could be present as thyroxin; from this quandary they find escape only in the suggestion that di-iodotyrosine in some specific combination in the thyroid-protein molecule has a calorigenic action little inferior to that of thyroxin. Canzanelli & Rapport find that thyroxin has 240 times the activity of free di-iodotyrosine (in dogs, injected intravenously) and 17 times that of di-iodothyronine; the last, however, is more active by mouth, being soluble (Anderson, Harington & Lyon). De Caro & Giani made similar comparisons in rats.

Lunde, Wülfert & Laland, and Barnes & Jones agree that most of the iodine and probably all of the activity may be found in the euglobulin fraction; according to Grab, a large fraction of the total iodine, and a still larger fraction of the total thyroxin may be found in the colloid of the gland.

It is usually felt that the thyroid hormone directly or indirectly stimulates all metabolic processes alike, yet there are some which appear to be specifically affected by thyroidectomy, such as the oxidation of purines (Flössner, Kutscher & Wittneben) and of acetonitrile (Baumann, Sprinson & Metzger).

Reviews of the whole subject have been published by Blanchard, Penau & Simonnet, Harington, and Trendelenburg & Krayer.

Insulin.—We have space here to discuss only three aspects of this subject. In the first place, mention should be made of the finding of Culhane & Greenwood and Hemmingsen & Marks that crystalline insulin assays higher by the mouse method than by the rabbit method; that is, it has more tendency to cause convulsions and relatively less tendency to lower the blood sugar than the international standard. Marks offers the interpretation that the relatively crude standard may contain impurities which delay recovery from hypoglycaemia in the rabbit test; it is probable that crystalline insulin will soon replace the present standard. Less comprehensible is Bronkhorst's claim that

certain very active preparations have little tendency to cause convulsions.

Depancreatized dogs receiving insulin and apparently in good condition, without fatty livers, are not normal in all respects: cholesterol esters are practically absent from the blood (Chaikoff & Kaplan), cataract is common (Chaikoff & Lachmann), lactation is inhibited (Chaikoff & Lyons), and, according to Ring & Hampel, nitrogen excretion remains high.

The control of insulin secretion remains a vexed question. There seems to be a response to hyperglycaemia within the pancreas (Kosaka), and vagotomy has no effect on sugar tolerance (Phillips, Högler), nor on glycogen restitution after exercise (Long & Fry); yet Geiger finds that the hypoglycaemia that follows warming of the carotid blood depends on the integrity of the vagi, and LaBarre & Vesselovsky found (though Gayet & Guillaumie did not) that vagal stimulation lowers blood sugar. Kuré *et al.* affirm that, when sympathetic effects are excluded by tying off the adrenals and painting the coeliac ganglion with nicotine, splanchnic stimulation lowers the blood sugar of vagotomized dogs, because "spinal parasympathetic" fibres evoke an insulin discharge.

Adrenal cortex.—In the period under review, this organ has been the subject of a somewhat unedifying controversy, which centres around the question of deciding which, if any, of the numerous symptoms of adrenal insufficiency is the primary one. Hartman, Brownell & Lockwood consider that the question is unanswerable and that "cortin" is required for the normal activity of many tissues. Swingle *et al.* lay all the emphasis on the anhydraemia, observed also by Corey & Britton (1) and others, and due possibly to escape of blood into the muscles (Silvette & Britton; Winter & Hartman). But Britton & Silvette feel that the reduced glycogen stores and tendency to hypoglycaemia indicate a severe primary breakdown of carbohydrate metabolism; Silvette affirms that cortical extracts accelerate glycolysis of blood sugar *in vitro,* but it seems doubtful whether this can be of physiological importance. Loeb *et al.* and Harrop *et al.* draw attention to the continued loss of sodium chloride in adrenalectomized animals as an important factor, while Rubin & Krick find that other inorganic ions are also lost and maintain that adrenalectomized rats survive indefinitely if a mixture of salts is supplied in the drinking-water. Firor & Grollman agree that adrenalectomy in the rat usually leads to death within a fortnight, but

those who have not encountered this phenomenon seem unwilling even to accept Gaunt's evidence that different rat strains behave differently. Gaunt & Gaunt find that even in susceptible strains many animals will survive indefinitely after a short period of treatment, during which accessories hypertrophy.

The lowered resistance of adrenalectomized animals has been reviewed by Scott *et al.* and by Perla & Marmorston, and studied with reference to cold by Baird, Cloney & Albright and by Martin & Maresh, with reference to fatigue by Eagle, Britton & Kline. Ingle, Csik, Ochoa and others have dealt with the rapid onset of fatigue in isolated muscles of adrenalectomized animals; Eagle's observation that most cortical extracts contain choline may be pertinent in this connection.

Adrenaline.—Kuschinsky & Viaud believe heretically that adrenaline restores the muscles of adrenalectomized animals; that it postpones fatigue to indirect stimulation in normal muscle was beautifully demonstrated by Corkill & Tiegs. Schild and Euler both disproved the assertion that a specially potent "novadrenine" is present in the medulla. Sato has reviewed some biological tests for adrenaline, and Barker, Eastland & Evers have re-examined the chemical tests, which have recently been added to by Viale, and Gaddum & Schild.

Using Viale's sensitive test, Bacq concluded that the substance liberated at sympathetic nerve-endings ("sympathin") was chemically similar to adrenaline, and Bacq & Henri found confirmatory spectroscopic evidence. But Cannon & Rosenblueth reached a different result; observing that in the spinal cat, sensitized with cocaine, stimulation of the sympathetic nerves to the duodenum (inhibitory) and liver (vasoconstrictor) causes by humoral transmission contraction of the nictitating membrane and relaxation of the uterus, but fails to produce the latter effect when the duodenal fibres are destroyed; and noting that when Bacq & Brouha stimulated the (vasodilator) sympathetic fibres in the sciatic they obtained inhibition of intestinal activity that was not seen after stimulation of (excitatory) sympathetic fibres to the tail, they conclude that sympathetic nerves of inhibitory function liberate "sympathin I" which affects only organs inhibited by the sympathetic, while excitatory fibres release "sympathin E," which likewise acts only on organs stimulated by the sympathetic. As mixed effects must predominate, it will, unfortunately, be difficult to put this interesting theory to numerous tests.

Acetyl-choline.—Humoral transmission of parasympathetic impulses by means of a substance indistinguishable from acetyl-choline can no longer be doubted; it has been demonstrated more or less conclusively, not only for the salivary glands, but also for the blood-vessels of the tongue (Bain; Feldberg; Plattner) and of the mesentery (Gollwitzer-Meier & Otte), and for the cardiac (Feldberg & Krayer) and the gastric vagus (Dale & Feldberg); acetyl-choline is normally present in portal blood (Feldberg & Rosenfeld). Chang & Gaddum have surveyed the acetyl-choline content of a variety of tissues; in very few (spleen, submaxillary gland, small intestine, stomach, bladder, auricle, and sympathetic nerve) is it present in concentrations exceeding one part per million, but human placenta is a relatively rich source, a fact also noted by Hauptstein and Chang & Wong, but of obscure significance.

Schilf & Sternberg noted that acetyl-choline has a nicotine-like stimulating action when applied directly to sympathetic ganglia; Kibjakow found that stimuli passing by pre-ganglionic fibres into the superior cervical ganglion of the cat could be humorally transmitted by the perfusion fluid to excite post-ganglionic fibres of another ganglion; and Feldberg & Gaddum found evidence that the active agent was acetyl-choline. Similarly Feldberg & Minz had found that acetyl-choline could liberate adrenaline from the adrenal medulla without nervous intervention, and Feldberg, Minz & Tsudzimura collected impressive evidence to show that acetyl-choline is an intermediary in the nervous stimulation of the adrenal medulla. It is likely that this rule may be generalized throughout the sympathetic system, but whether parasympathetic preganglionic fibres liberate substances at their synapses is as yet undetermined.

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VITAMINS*

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The space allotted has enabled us to deal with no more than about one quarter of the total number of papers published during the year. It deserves to be said that of the large number thus crowded out the great majority represent some definite addition to knowledge, filling in some detail or other on the big canvas. We make this point because superficial critics so often suggest that out of this immense annual output of papers only comparatively few can be of real permanent value. This criticism seems to the reviewer to overlook the essential fact that (as all past experience goes to prove) progress is made only by the cumulative and co-operative efforts of many different workers, each adding his contribution to the general flow of knowledge. Solitary isolated advances are few. And much work is nevertheless useful and essential, although it represents no fresh development of theory, and finds no place in our review—e.g., determinations of the vitamin resources of different parts of the globe, assays of vitamin distribution on many dozens of different foodstuffs, technical studies on the effects of different commercial processes, clinical investigations on the prevalence of hypo- and avitaminosis in various regions, much detailed, descriptive, histological, and pathological work, and agricultural applications—while even purely confirmatory work has its value.

Among the most important developments of the year are the synthesis of ascorbic acid and determination of its structure, and the demonstration of the vitamin-B_2 activity of flavine. Whether flavine is the antipellagra factor or whether (as seems likely) some other constituent of a complex B_2 remains to be shown.

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PART I. WATER-SOLUBLE GROUP

VITAMIN B_1

Vitamin-B deficiency in human beings.—Few Western biochemists realise the extraordinary prevalence of vitamin-B deficiency in many Eastern countries. An illuminating paper by Balfour & Talpade draws one's attention to the fact that in Southern India, where milled rice is the staple diet, nearly all pregnant females are in a state of avitaminosis. As a result the incidence of premature births is three times as great as it is in the north of India (where wheat is the staple diet), and in consequence the infant-mortality rate also is many times greater. As a practical remedy at least 8 ounces of wheat daily must be added to the diet.

In Japan, 17,000 deaths are said to occur from beri-beri every year according to *Science News Letter.*

A good deal of evidence has now been acquired to show that even under Western conditions much would probably be gained if all infants were allowed special supplementary sources of vitamin B, in much the same way that it is already the orthodox procedure to provide routine additions of vitamin C (orange juice) and D (cod-liver oil). We have to recognise that milk is not a very rich source of vitamin B and there are suggestive indications, emanating mainly from America, of the surprising improvements in weight and height gains which may result with extra vitamin B (see reviews by Harris and Sure).

Physiology of vitamin B_1 action.—The evidence continues to grow that vitamin B_1 is concerned in some oxidative mechanism in carbohydrate metabolism. Additional studies have appeared from Peters' laboratory on the oxygen uptake of brain from avitaminous pigeons. This tissue, when minced and mixed with lactic acid, shows a low oxygen uptake compared with normal pigeon brain, and the uptake can be improved by addition of vitamin-B_1 concentrates *in vitro* [Meiklejohn, Passmore & Peters (1); Passmore, Peters & Sinclair]. The position becomes more obscure, however, and gains in complexity by the later surprising finding that the actual amount of lactate removed during the oxidation is not influenced at all by the presence of the vitamin, in fact the avitaminous brain itself without any addition of vitamin B is already able to remove the lactate *in vitro* (Meiklejohn). A further puzzle is that, while avitaminous brain tissue shows a low oxygen uptake with pyruvic acid similar

to that with lactic acid, in this case the addition of vitamin B_1 is without any effect [Meiklejohn, Passmore & Peters (2)]. Clearly much further work is needed to give the explanation of these differences.

A very attractive hypothesis in accordance with many known facts is that vitamin B_1 enters into the mechanism of an oxidative enzyme system, such as a dehydrogenase system, in a rôle corresponding to that of co-enzyme. More specifically the theory may be put forward that vitamin B_1 is a precursor and component of such a factor, in the same way that it now seems clear that vitamin B_2 (as flavine) comprises a constituent of Warburg's yellow oxidation enzyme. Boyland has examined the theory that vitamin B_1 is the same as Banga & Szent-Györgyi's co-enzyme for lactic-acid dehydrogenase; but finds that, although crude vitamin-B_1 preparations do indeed possess co-enzyme activity, more purified ones do not, and the purified co-enzyme is likewise without vitamin-B_1 activity. Some close interrelation nevertheless seems likely with it and with the "vitamin-B_4" fraction. The present writer had come to pay special regard to three salient facts as of significance for vitamin-B_1 action. It was therefore a striking coincidence when these same three points were also seen to come prominently into the picture in Szent-Györgyi's co-enzyme. These three points are (*a*) an association, topographically, with heart muscle, (*b*) physiologically with the disappearance of lactic acid, and (*c*) an association chemically with adenine in combination with sulphur. To be more explicit: (*a*) In the case of the vitamin, work by Drury and the writer showed that vitamin B_1 was essential for maintaining a normal rate of heart beat (preventing bradycardia) in the rat. (*b*) Later work showed that the bradycardia went with an increased concentration of lactic acid and the cure with its removal (Birch & Harris). (*c*) The tendency for a chemical association or adherence between adenine and vitamin B_1 (however accidental it may be regarded) is well illustrated by such facts as the following: e.g., that irradiated adenine sulphate, although it is not identical with vitamin B_1, is nevertheless said to possess a vitamin-B–like growth-promoting action (Guha & Chakravorty), and that vitamin B_4, in turn, a deficiency of which leads to symptoms so generally similar to those of vitamin-B_1–deficiency and which is also said to display its activity *in the actual crystalline preparations of vitamin B_1 itself*, is found to be associated, in its most concentrated preparations, with adenine in a relatively pure state (see below under

vitamin B_4). The co-enzyme, on its side, is (*a*) likewise needed for the normal functioning of the heart muscle and it is from this source that it has been isolated; (*b*) it is concerned in removing the lactic acid; and (*c*) the purest preparations are said to consist of a sulphur-containing adenine derivative. Both vitamin B_1 and the enzyme preparation contain sulphur, and both are basic and tend to come down with adenine fractions. The nature of the interrelation can be cleared up only by further observations; in the meantime it is obvious of course that the elementary analysis of adenine itself bears little resemblance to that of crystalline vitamin B_1. The "vitamin-B_4 fraction" seems incriminated, and we may note further that the importance of adenine derivatives for the physiology of the heart beat is already recognised.

Simola has found that there is no dependence between vitamin B_1 and co-zymase, the co-zymase activity of liver or brain remaining unchanged in avitaminosis. He makes a suggestion however of a possible decreased co-carboxylase activity in avitaminosis B_1.

Vitamin B and the dietary balance.—Two further papers by Evans & Lepkovsky report that the "vitamin-B–sparing action" caused by an increased allowance of fat in the diet can still be demonstrated when the vitamin B is given intraperitoneally or when glycerides of purified oleic acid are used as sources of fat (in place of a natural fat). Although Gregory & Drummond could not confirm this sparing action of fat, Birch has been able to do so.

The theory of Drummond that an increased proportion of protein needs balancing by an increased percentage of vitamin B in the diet has been supported by Bell from Drummond's laboratory, but to judge from Prunty & Roscoe's results there is in fact no real relation between the protein intake and the actual vitamin-B requirements. The addition of excess of protein to a diet merely acts indirectly by reducing the food consumption. When, to obviate this complicating factor, the vitamin B is given separately, instead of as a percentage of the diet, the balance effect is no longer apparent.

Isolation of vitamin B_1.—The previous indications that the crystalline specimens isolated by Jansen & Donath, by Windaus, by Peters, by Ohdake, and others are all substantially identical is supported by the findings of Bernal & Crowfoot, who have examined the crystal form and X-ray pattern of four different specimens obtained from three of the above-mentioned workers (viz., Jansen & Donath, Windaus & Tschesche, and two specimens from Peters).

Van Veen also has isolated a crystalline preparation which he finds to have the same properties as Windaus' and Ohdake's. He gives the rat dose as 4–15γ; Ohdake, also, with conventional methods [precipitation of oryzanin extract with $AgNO_3$ and $Ba(OH)_2$ followed by phosphotungstic acid and separation of Pt salt and hydrochloride], obtained a preparation with formula $C_{12}H_{16}N_4O_2S.2HCl$. Windaus, Tschesche & Ruhkopf likewise confirm the idea that the several preparations from yeast and rice bran are identical; analysis of the picrolonate hydrochloride, chloraurate, and rufianate, however, led to the slightly different formula $C_{12}H_{16}N_4OS$ for the free base (cf. Jansen, Wibaut, Hubers & Wiardi, who give $C_{12}H_{18}N_4O_2S.2HCl$ for the hydrochloride). Peters and co-workers, on the other hand, maintain that their own preparation is significantly more active than other workers had claimed theirs to be, viz., they found the average pigeon dose to be 2.17γ (given by mouth) compared with 2.4γ reported by Windaus (given by injection), or "of the order" 2.5γ by Ohdake. They add: "It is certainly a striking circumstance that our values should be so near . . . . Nevertheless we consider this to be a coincidence." The vitamin-B_4 activity of their crystals was also taken to argue against their purity, and "this leads to the unfortunate conclusion that neither ourselves nor others have yet reached pure vitamin B_1" (Kinnersley, O'Brien & Peters).

Concentration methods.—Seidell & Smith have described a more efficient and direct procedure in place of the usual tedious and wasteful processes. It has the further advantage of giving much larger yields (viz., 25 per cent). The final isolated product had the same activity as that of Jansen or Windaus or Peters (curative rat dose given as 0.008 to 0.012 mg.). The relatively short process described involves adsorption on fuller's earth followed by elution, benzoylation, and precipitation with acetone, and fractional crystallisation with picrolonic acid and recrystallisation from methyl alcohol. The Oxford workers have given full details of the large-scale application of their method which takes fourteen days for completion (Kinnersley, O'Brien, Peters & Reader; Kinnersley, O'Brien & Peters).

Block & Cowgill describe a very simple process for concentrating the vitamin up to a potency equivalent to that of Jansen & Donath's Pt-stage (0.1 mg. pigeon dose). This depends on extraction of the vitamin from alkaline solution in organic solvents (based on the theory of its basic nature). Yields of 90 per cent can be obtained instead of about 5 per cent by the more usual elaborate procedures.

Physico-chemical properties.—The ultra-violet absorption spectrum of crystalline vitamin B_1 has been examined by Peters & Philpot, who find it to have a maximum at 245–249 mμ: reversible changes occurred in the spectrum on alkalisation.

The heat stability has been further studied by Elvehjem, Kline, Keenan & Hart, who have made the unexpected discovery that presence of moisture greatly accelerates the rate of destruction, the potency not being affected even by 24 hours heating at 100° in the dry state.

Adsorption experiments by Sherman & Halliday show that Lloyd's reagent adsorbs half of the vitamin-B_1 activity and one-third of the B_2 activity from protein-free milk, yet the filtrate which remains shows no vitamin-B_1 activity and only one-sixth of the original B_2 activity.

Methods of assay.—A very detailed report from the pharmaceutical laboratories by Coward, Burn, Ling & Morgan mentions that in the pigeon test the probable error may be between the limits −23 to +31 per cent, and with rats −5 to +6. Most workers have been troubled by the often erratic biological variation between individual pigeons, but these workers propose to estimate vitamin-B_1 activity from the percentage of birds which are found to remain cured or uncured in a given test. With certain sources of the vitamin they had the disturbing experience that the rat dose did not run strictly parallel with the pigeon dose. They also found that the dose-effect curve was curvilinear, so that under some circumstances the assumption which is often made that the duration of the cure is proportional to the size of the dose may be only approximately true.

Passmore, Peters & Sinclair show that the oxygen uptake of avitaminous pigeon brain in co-ferment tests runs parallel with the amount of vitamin B_1 added, and they suggest the use of this reaction as a possible chemical test for vitamin B_1.

Distribution of vitamin B_1.—About 60 papers have appeared during the year dealing with the occurrence of vitamin B in various foods, or with the natural resources of different countries and states, or the effect of various treatments and manufacturing processes.

According to a comprehensive series of tests by Plimmer, Raymond & Lowndes (by their pigeon protective method) a number of animal foodstuffs (liver, kidney, heart, hard roe, skim milk, egg yolk) are found to contain more vitamin B_1 than fruit or vegetables in the fresh state, but less than cereals or pulses; while on the dry-

weight basis they may exceed cereals or pulses. Reference must be made to their original tables for the full comparative values.

It is disconcerting to learn from Seidell that specimens of commercial dried brewers' yeast may vary as much as tenfold in activity.

Various milk products are relatively richer in B_2 than B_1 (Bender & Supplee). Egg yolk is richer in B_1 and the white in B_2. The B_1 potency, at any rate, can be altered considerably according to the diet of the hen (Ellis, Miller, Titus & Byerly).

A careful comparison of raw and pasteurised milk by Krauss, Erb & Washburn of the Ohio Agricultural Experiment Station leads to the surprising finding that some 25 per cent of the vitamin B_1 is destroyed by the pasteurisation process. Vitamins B_2, A, and D were unaffected: it is of course already known that vitamin C may suffer some destruction. The so-called "physiological control" of the vitamin-B value of milk is not yet fully understood. Sherman believes that it cannot readily be raised above a normal value by the feeding of extra vitamin to the cow, and that, while it can be artificially lowered by the feeding of rations very poor in vitamin B_1, it is quickly brought back to normal once the cow resumes a normal diet. Schieblich however considers that cows, or ruminants in general, are able to synthesise vitamin B internally by bacterial activity, irrespective of the external sources.

Vitamin B_2

Symptoms of avitaminosis-B_2 in experimental animals.—The conditions which determine whether rats deprived of vitamin B_2 will develop the characteristic skin lesions or remain free from them are not yet properly understood. Hogan & Richardson have given a valuable account of the dietary conditions under which in their hands 100 per cent of rats are found to develop the lesions. The special feature is the irradiation of the diet, a possible meaning for which now becomes apparent, since vitamin B_2 (or one of its main constituents) has been identified as a flavine pigment which, as is known, is readily destroyed by the action of visible light. For some reason dermatitis did not appear in Hogan & Richardson's experiments when maize starch was used in place of sucrose in the basal diet, and the presence of butter or lard or Crisco sometimes prevented it also. At the Lister Institute the records show that no less than 108 out of 191 rats on vitamin-B_2–free diets during the past

seven years have developed dermatitis [Roscoe (1)]. Usually a period of 10 weeks was needed for its development and numerous variations in the composition of the basal diet and in the consequent growth responses were all without influence on its incidence. Other workers have been unable to hit on the right accidental conditions. It may be noted in particular that in the actual isolation work of Kuhn and collaborators in Germany the vitamin-B_2–deficient animals generally remained free from dermatitis. We shall have to return to this anomaly later.

An observation which deserves further investigation is that of Langston & Day on the production of keratitis and cataract in rats fed on diets deficient in vitamin B_2. This might be borne in mind in conjunction with the clinical records of corneal lesions seen in female beri-beri patients at pregnancy (Ayuyao).

It has been found that chicks, in contrast with pigeons, apparently need vitamin B_2 (Kline, Keenan, Elvehjem & Hart).

Pellagra in humans.—Much discussion is still devoted to numerous theories attributing the cause of pellagra to various other factors, apart from vitamin deficiency. The toxin theory has been revived most recently, surprisingly enough, by a vitamin worker (Chick), who postulates the presence of a pellagra-producing toxin in maize, in order to account for the "almost invariable association of pellagra with maize." As the *Lancet* has pointed out, however, there is no secure experimental basis for this view; and it may be stated, moreover, that pellagra is in fact not infrequently seen on a maize-free diet. For example, a series of 51 typical cases reported from Denmark (Thaysen) show how pellagra may be found, when it is looked for, in the most unsuspected places. An instance in a village in Cambridgeshire, England, may be cited where the diet was definitely maize-free (Cole). Ellis emphasises once again how frequently pellagra may be seen on maize-free diets, a definite aetiological group of cases being due to faulty absorption from the intestinal tract, whether caused by chronic diarrhoea resulting from colitis or tuberculous enteritis, or even coeliac disease. Alcoholism, as is well known, may have the same result.

Stockman & Johnston have reported that a disease having the pathological characteristics of pellagra was produced in monkeys, rabbits, or guinea-pigs whenever the diet was rich enough in cereals, and therefore they discount "the vitamin hypothesis" and would regard excess cereal *per se* as the cause. Wilson, on the other hand,

still upholds the protein theory; while Spies describes an experiment in which he claims that pellagrins recovered on a diet actually poor in vitamins, such as is actually used to induce pellagra in rats. It seems to the writer, however, that these theories all fail in neglecting to take into account the classical observations of Voegtlin and of Goldberger that pellagra could in fact be cured by the administration of a protein-free (and amino-nitrogen–free) vitamin extract.

In Egypt pellagra is still "one of the very common endemic diseases" (Biggam & Ghalioungui). Its incidence is almost entirely confined to the fellah (farmer) class; Sandwith makes the pessimistic comment that after ten years intense study of the subject, 62 per cent of the population still have some degree of pellagra. The exact incidence figure is put rather lower by others. In the United States, Wheeler & Sebrell, of the Public Health Service, mention that the number of deaths from pellagra actually included in official certificates for 1930 was no less than 7,146. According to the statistics of Petersen, 10,663 deaths were registered in 1915 for example, while the real number of deaths from the disease (many not being included in official returns) must have been nearer 16,000 or 20,000. There has been some dispute (Hoffman; Harris) as to whether or not the statistics, to be found at the Census Office at Washington, give an accurate representation of the full death-rate, since the maximum shown there for any single year is 6,989; but the conclusion seems indisputable that the disease is preventable and due to inadequate diet and many thousands of unnecessary deaths occur annually at a time when output is being artificially restricted and food burned or thrown into the sea.

Flavine as vitamin B_2.—The outstanding event of the year has been the identification of vitamin B_2 (or a major component) as the naturally occurring water-soluble pigment, first found in milk as "lactochrome" (now renamed "lactoflavine") by Winter-Blyth as long ago as 1879 and further studied by Bleyer & Kallman (1925) and others. The flavine present in egg-white (ovoflavine) appears to be identical chemically with that in milk (lactoflavine) and to be equally active biologically, and there can be little doubt also that the flavine occurring in other tissues, such as, e.g., muscle, is equally the source of vitamin-B_2 activity; whether there may be any minor variations in chemical configuration or in biological potency of the pigment as it occurs in different sources remains to be established.

The discovery of the vitamin activity of the flavines is due to

Kuhn, György & Wagner-Jauregg, from whom a series of four papers on this question has appeared. Booher has published independent work in excellent confirmation; and Karrer & Euler have brought forward further supporting evidence. In their first preliminary note Kuhn, György & Wagner-Jauregg (1) describe how when a vitamin-B_2 concentrate is sufficiently purified it is found to possess a bright yellow colour and a characteristic intense green fluorescence. The method of concentration adopted involved adsorption from acid solution on fuller's earth, elution with dilute pyridine or ammonia, followed by removal of impurities by precipitation with alcohol and acetone. Each increase in biological activity was found to run parallel with the increase in yellowness and green fluorescence. To procure reasonable growth responses in rats already receiving separate supplements of vitamin B_1, as much as 500γ of the yellow preparation were needed, although some growth restoration resulted from 100γ; but, as later papers indicate, a much smaller dose suffices once provision is made for supplementing the diet with "vitamin B_4" also. The second paper [Kuhn, György & Wagner-Jauregg (2)] describes in detail the procedure for extracting the flavine from egg-white; it is described as "one component of the vitamin-B_2 complex"; and the probable formula $C_{16}H_{20}N_4O_6$ or $C_{17}H_{20}N_4O_6$ is assigned to it. In a further communication [Kuhn, György & Wagner-Jauregg (3)] the properties of lactoflavine as prepared from milk are described. Judged by its identical crystalline form, absorption spectrum, and elementary analysis ($C_{17}H_{20}N_4O_6$), the lactoflavine appeared to be identical with ovoflavine. With diets supplemented with both B_1 and B_4, the rat dose was 5γ per rat per day for a weight increase of 40 gm. in four weeks.

The principal results of these investigations have been conveniently summarised in two short papers by György, Kuhn & Wagner-Jauregg. In the first of these the interesting fact is disclosed that one starting-point of the investigation was the observation that the dark-coloured leg muscles of the fowl contained more vitamin B_2 than the lighter-coloured heart muscle. An endeavour to trace the relationship showed that the pigment in question was not a haemin derivative, and further inquiry established the association of biological activity with the yellow colour and green fluorescence. Vitamin-B_2 activity, like the flavine, is destroyed by exposure to visible light (in the blue or violet). Both ovoflavine and lactoflavine are relatively

inactive until vitamin B_4 is added, but the conclusion seems justified that the flavine is a major component of the B_2 complex.

Independent work on the isolation of vitamin B_2 from whey, by Booher, has led to results in striking confirmation of those reported by Kuhn, György & Wagner-Jauregg (1, 2). Booher's procedure, following along the lines of previous work at the same laboratory by Page, was to restrict herself to relatively mild operations and to avoid precipitation reactions; by such method she was able finally to achieve a 2,000-fold increase in potency compared with the original whole milk. Essential steps in the process are extraction of the whey powder with boiling ethyl alcohol, washing the dried extract with a mixture of chloroform and ethyl alcohol, and extraction of the residue with ether.

Booher describes her preparation as a solid, red hygroscopic substance, which is soluble in water or 94 per cent hot alcohol or chloroform-alcohol mixture to yield a yellow solution with a green fluorescence. It is readily decolorised by sunlight. The colour is increased in alkaline reaction and disappears in acid. Analyses show the presence of sulphur (cf. Kuhn *et al.*) and nitrogen. The biuret reaction is negative, but silver nitrate gives a precipitation. Absorption is general between 2,000 and 5,000 mμ. It is concluded that the substance is similar to ovoflavine or the lactochrome of whey described by Blyth (1879) and later by Bleyer & Kallmann (1925) and others. It had vitamin-B_2 activity in doses equal to or rather less than 1 mg.

Results similar to those of Kuhn, György & Wagner-Jauregg are published also by Karrer & Euler. They describe the separation from aqueous liver extracts of an "Fl" fraction, possessing an ultra-violet absorption maximum at 260 mμ and also absorption in the visible range. The rat dose was 25–50γ, for a given gain of 0.6–0.9 gm. per day, but better responses occurred when a separate supplement of vitamin-B_2–free malt extract was given, which seems comparable with the effect of supplements of vitamin B_4 noted by György, Kuhn & Wagner-Jauregg (2).

Vitamin B_2 and Warburg's yellow oxidation enzyme.—One question of great importance discussed by György, Kuhn & Wagner-Jauregg is the relation of the flavine to the "yellow oxidation enzyme" of Warburg & Christian. The matter may be summed up briefly by saying that the yellow enzyme consists of the flavine in combination with a colloidal grouping. The flavine, both as it occurs separately

and also in the combined state in the enzyme, possesses vitamin activity; whereas the free flavine itself, of course, has no enzyme activity. Boiling the enzyme destroys its activity *qua* enzyme, but not its vitamin function. These two forms of the flavine, in its simple state and combined as enzyme, occur together naturally, and can be separated one from the other by dialysis.

The importance of this relationship for an understanding of the physiology of vitamin action is this: that it appears to indicate that vitamin B_2 is needed for the elaboration of this essential enzyme, which the animal organism by itself is unable to synthesise.

Bierich, Lang & Rosenbohm have already made a potentiometric study of this "reversible, yellow, fluorescent oxidation-reduction system in the tissues of animals, which may be identical with the Warburg-Christian yellow oxidation enzyme and with vitamin B_2." It is noteworthy that they find that the E_0 value is lower than that of any physiological system previously known.

Vitamin B_2, flavine and dermatitis.—Is the flavine identical with the anti-dermatitis or pellagra-preventive principle, or is it just one of a number of growth-promoting factors present in a complex and composite vitamin B_2? Several suggestive facts might be brought forward to support the theory of the identity of flavine with the "P-P" substance. Thus, it is known that irradiation of a diet confers dermatitis-producing properties on it, and it would seem reasonable to link this with the fact that such treatment does in fact rapidly bleach and destroy the flavine. Again the seasonal incidence of or "photo-sensitisation" to pellagra might, I would suggest, be explained to some extent by the destruction of the flavine by the action of bright sunlight, either on the natural food materials or, *in vivo*, on subjects exposed to it. Several puzzling difficulties have still to be accounted for, however. As already mentioned, György, Kuhn & Wagner-Jauregg (2) very rarely saw symptoms of dermatitis in their flavine-deficient rats and the suggestion was thrown out that there might be some new factor, "vitamin H," as the antidermatitis constituent of the vitamin-B complex. On the other side Roscoe (2) found that dermatitis prevention went parallel with growth promotion in the various sources of vitamin B_2 which she examined, and concluded therefore that there was "no support for the theory of separate factors for dermatitis-prevention and growth." It may be suspected that some small technical differences in details

of procedure not yet fully understood are responsible for this lack of conformity between the results of different workers.

Vitamin B_4

The announcement of the isolation of a crystalline preparation of vitamin B_4 by Barnes, O'Brien & Reader was followed before many weeks had elapsed by the unexpected disclosure by Tschesche that the crystals in question consisted essentially of adenine hydrochloride. This has been confirmed by Bernal & Crowfoot's crystallographic data; but since *pure* adenine hydrochloride itself is inactive it is now thought by the Oxford workers that the activity of the crystalline adenine preparation must be due to the presence in it of traces of some impurity (Heard, Kinnersley, O'Brien, Peters & Reader). Another possibility is that in the potent crystals adenine may be present in some specially activated form. The understanding of the position is of course complicated by such considerations as the following: (*a*) that the apparently pure crystalline preparation of vitamin B_1, as isolated independently in different laboratories, is one of the richest sources of vitamin-B_4 activity; (*b*) that by definition the symptoms of vitamin-B_4 deficiency bear a general resemblance to those of vitamin-B_1 deficiency; and (*c*) that the standard procedure for producing avitaminosis-B_4 (as of measuring B_4 activity) actually consists in first subjecting the experimental animals to vitamin-B_1 deficiency. Needless to say, these facts do not throw any doubt on the reality of the actual phenomena described: they only indicate the complexity of the problem and suggest the probability that there is some kind of interconnection or close association not yet understood between vitamins B_1 and B_4.

The isolation process described by Barnes, O'Brien & Reader (1, 2) involves adsorption on charcoal at selected pH values, extraction with acid alcohol, and successive treatment with $HgSO_4$, $Ba(OH)_2$, and Na phosphotungstate, and removal of a pentose group by acid hydrolysis. The crystalline material was given the formula $C_4N_4H_5Cl$, or $C_4H_4N_4.HCl.\frac{1}{2}H_2O$. The rat dose was 0.01 mg. per day. Details of procedure suitable for a large-scale operation have been published also (Kinnersley, O'Brien, Peters & Reader). Confirmatory evidence of the necessity for a third factor (vitamin B_4) for rats allowed only limited amounts of vitamins B_1 and B_2 is provided by Moore, Plymate & Andrew.

Other "B" Factors

Vitamin B_3.—A number of workers have sought in vain to obtain confirmatory evidence of the existence of a "vitamin B_3," distinct from the purely antineuritic B_1. Morris found that the alleged growth-promoting action of vitamin B_3 seemed in reality to be but the effect of giving a more adequate allowance of B_1, and he was unable to distinguish any difference in properties such as heat stability between B_1 and B_3, or to separate them. Graham & Griffith had a rather similar experience and refer also to the conclusion of Hunt that B_3 appears to be "a more abundant supply of B_1." Elvehjem and co-workers also were unable to find that chicks need a heat-labile vitamin B_3.

Unclassified.—The vitamin-like growth-promoting action of irradiated adenine sulphate for rats on synthetic diets, apparently complete in B_1 and B_2, is described by Guha & Chakravorty; the earlier suggestion of actual antineuritic action is withdrawn and is negatived by work elsewhere (Schultz & Laquer; Yamamato & Yamagishi).

A new paper by Mapson describes the striking symptoms, leading to death, seen in the offspring of rats fed on a certain type of purified casein ("Glaxo Casein") as their source of protein. The condition is alleviated by physin[1] and probably by the "Coward Factor," and the possible identity of the two is discussed.

McCay and co-workers find that trout fail to thrive when provided with diets containing adequate amounts of vitamins A, B, C, D, and E and other known essentials. Raw liver or preserved raw meat rectify the deficiency and are postulated to contain a new factor, "H."

Micro-Organisms and Vitamins

Again this year a large literature has appeared describing, on the one hand, the synthesis of vitamin B or similar growth factors by various species of micro-organisms, and on the other the requirements of the micro-organism itself for growth-stimulating substances which need to be present in the media in which it is grown.

Miller, Eastcott & Maconochie after reviewing past work on the fractionation of bios into two constituents, Bios I (*i*-inositol) and

[1] Cf. *Ann. Rev. Biochem.*, **2**, 261 (1933).

Bios II, proceed to describe the fractionation of the latter into two portions, which they call Bios IIA and Bios IIB.

Williams and his collaborators have shown that tissue extracts prepared from a great diversity of different species, both animal and vegetable, are all able to stimulate the growth of yeast. They suppose that the effect is due to one single substance, "of universal biological occurrence," which they call *pantothenic acid* (Greek "from everywhere"). The supposition is based on the similarity in behaviour of the extracts prepared from the different tissues in question. The substance is thought to have a molecular weight of the order of 150, and to be an acid containing several hydroxyl groups; but it contains no olefine, keto, sulphydryl, amino, aromatic, or sugar groups, and (it is suggested) bears a close relationship to vitamin B_2.

The phenomenon of refection seems to be produced more readily in some laboratories than others, depending no doubt on the local micro-flora. Thus we have always been able to produce it at will in the Cambridge Nutritional Laboratory, whereas many have tried without success hitherto in the United States. It is of interest therefore to note a recent paper by Parsons, Kelly & Hussmann describing an authentic instance of refection, seen in the University of Wisconsin.

Since it is known that plant organisms synthesise only carotene and not vitamin A (animal species converting this carotene into vitamin A), much interest attaches to the finding of Baumann, Steenbock & Ingraham that certain bacteria fall into their due position in the vegetable kingdom.

Vitamin B and Anaemia

A current problem of much theoretical interest, as well as of practical clinical importance, is the nature of an anti-anaemic principle known to be present in certain materials rich in the vitamin-B complex, and the question of its relationship to the known members of the complex. The striking clinical work of Lucy Wills (1) has demonstrated the value and specificity of a vitamin-B preparation such as "marmite" (an autoclaved yeast extract) in curing cases of tropical macrocytic anaemia, including especially the so-called "tropical pernicious anaemia of pregnancy." A suggestion that the active principle in the marmite might be vitamin B_2 has been disproved, as Wills (2) has now shown that preparations containing vitamins B_1, B_2, or B_4 were all without curative action. The identity of the anti-

anaemic factor in question is, then, still hidden, but it is known to be heat-stable, soluble in water and in 80 per cent alcohol. The work of Guha on haemopoiesis in rats likewise indicates its nonidentity with vitamin B_2. A further advance, which gives added conviction to the whole phenomenon, is the production of macrocytic anaemia in experimental monkeys by feeding them on those same diets on which women in India actually contract this anaemia of pregnancy (Wills & Bilimoria). Following on Wills's work, vitamin-B therapy ("marmite") has also been successfully applied to the macrocytic anaemia of coeliac disease (Vaughan & Hunter) and in sprue (Strauss & Castle; Castle & Rhoads).

For many other types of anaemia vitamin B is of course without effect. Thus it is no substitute for liver in pernicious anaemia, notwithstanding some partial successes reported by Goodall, by Ungley, by Castle, and by Davidson. [The specific substance curative of pernicious anaemia, although it occurs associated with vitamin B_2 in the crude state, can be obtained, repurified, quite free from it (Brand, West & Stucky).] Again, vitamin B_2 from egg-white is without action in pernicious anaemia (Wills & Naish), which disproves the theory of Strauss & Castle that vitamin B_2 might be the "extrinsic factor" which on interaction with the "intrinsic factor" present in normal gastric juice (but absent from cases of pernicious anaemia) is supposed to give rise to the haemopoietic factor of the liver. Vitamin-B therapy is likewise without specific action in adult hypochromic anaemias or microcytic anaemias generally, or in simple nutritional anaemia, although it has been suggested that in the latter condition it may help to supplement the action of iron to some extent (Parsons & Hickman).

Vitamin B and glossitis. — Communications from two sets of workers have demonstrated the probability of some important relationship between a constituent of the vitamin-B complex and glossitis (with associated anaemia). Miller & Rhoads describe how dogs kept on a diet low in vitamin B_2 acquire symptoms resembling sprue. A large percentage develop stomatitis and glossitis and gastro-intestinal upset, and often anaemia, in some of the more severe cases of which histological changes in the bone marrow may occur similar to those seen in clinical sprue or in Addisonian pernicious anaemia. Clinical trials on human subjects suffering from glossitis proved the beneficial effect of yeast when combined with liver therapy. Yeast alone was not always satisfactory. The second paper referred to, by Hutter,

Middleton & Steenbock, describes the occurrence of glossitis in rats on vitamin-B–deficient diets. These interesting developments seem to indicate that the glossitis of sprue and of Addisonian pernicious anaemia in man may very well be caused by lack of some part of the vitamin-B complex.

Relation of iron to the nervous manifestations in pellagra and combined degeneration.—Recently Sargant made the surprising finding that nervous manifestations in cases of sub-acute combined degeneration of the cord (e.g., in Addisonian anaemia) were greatly ameliorated by massive doses of iron. The improvement was not necessarily limited to anaemia but was sometimes seen in cases in which there was no apparent abnormality of the blood picture (Harris & Sargant). Acting on the similarity of the nervous lesions in combined degeneration with those in pellagra, Biggam & Ghaliounqui have now tested massive iron therapy in cases of pellagra showing nervous manifestations, and have made the important observation that such treatment brings about a very much more rapid improvement in the patient's condition than when the ordinary dietary treatment alone is given. We have in these findings yet a further example of the effect of a vitamin-B deficiency in bringing about some error in iron metabolism, of a nature not yet clearly understood.

Vitamins and Choice of Diet

Experiments carried out over a period of years at Cambridge (Harris, Clay, Hargreaves & Ward) have investigated the question of the extent to which animals are able to choose "by instinct" between adequate and deficient dietary constituents and, as a corollary, the nature of the mechanism by which in certain cases the correct choice is made. The general conclusion reached is that an essential factor enabling the animal to make the correct choice is the past experience of the beneficial effects following on the consumption of the right diet, i.e., the "reward." There is no generalised instinct, or "food conscience," to guide between adequate and inadequate diets.

The experimental observations are too lengthy and varied to be reviewed adequately here, but the following gives an indication of the general technique adopted. Rats depleted of vitamin B (or B_1) were found always to choose a diet containing the vitamin rather than the same diet without it, provided that they could distinguish between the two by smell or taste, or sometimes appearance. For this purpose the vitamin had to be incorporated in a readily recognisable form such

as yeast extract or wheat germ; if it were given as a minute amount of a potent concentrate the choice could not be made. Rats not depleted (or dosed separately with the vitamin) ate indiscriminately, until they began to suffer from the deficiency, when the correct choice was made. When the depleted rats were offered a sufficiently large choice they were unable to select that containing the vitamin, but if they were now "educated" for a day or so on this diet alone they were then able to choose correctly from the series. But if at this stage the vitamin were changed over, leaving the taste and smell of the various diets unaltered, the rats were then "deceived" and unsuspectingly went on eating the now deficient diet. They could then be "re-educated" afresh to choose the diet now containing the vitamin. This last experiment in particular led to the conclusion that "this ability of the depleted rat to discriminate between diets containing and deficient in vitamin B depends not on vague instinct but on an association between the distinctive character of the diet (smell, taste, or appearance) and the prompt beneficial effects, e.g., on appetite, alimentary tone, etc., which immediately follow its consumption." This conclusion is substantiated by the experiments with other dietary essentials such as vitamin A, vitamin D, and protein, whose effect is less immediately apparent, and in which case it was found that the rats were unable to choose. Animals are therefore not "dietetic experts by nature." The correct choice may be made for a constituent that does good rapidly, but there is no guide at all for others which, although just as important ultimately for the well-being of the individual, are slower in action. Various implications of this work in its different aspects have been discussed by other writers [including certain philosophical deductions (cf. Bacharach)].

Vitamin C

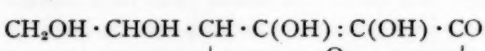
Specificity of ascorbic acid as antiscorbutic factor.—Last year's report related how Tillmans and his co-workers in Germany had concluded that vitamin C was identical with a reducing principle present in plant sources, probably the same as Szent-Györgyi's "hexuronic acid," how King in America having isolated a crystalline preparation of vitamin C from lemon juice found it to have the properties of hexuronic acid, and how Szent-Györgyi, testing hexuronic acid, found it to possess antiscorbutic properties. The theory that hexuronic acid [since renamed ascorbic acid (Szent-Györgyi & Haworth)] was identical with vitamin C was disputed by Zilva. Tests were car-

ried out in the writer's laboratory and elsewhere to determine whether the activity of ascorbic acid was indeed an inherent property of the pure substance itself or due to some associated impurity. The results of a very elaborate enquiry [Harris & Ray (3); Birch, Harris & Ray (1)] showed that there was in fact an unvarying correlation between vitamin C and ascorbic acid, so that no doubt could remain as to the specificity of the substance. For example, it was shown that the biological activities of some forty different sources could be accurately predicted from their ascorbic acid contents as determined chemically. Sources not previously recognised as containing the vitamin were brought to light in this way, and the activity afterwards confirmed biologically. Again the synthesis of vitamin C in nature, in the vegetable kingdom as well as in certain animal species, was found to go side by side with the amount of ascorbic acid formed. Vitamin-C activity disappeared from animal tissues during scurvy concurrently with the disappearance of ascorbic acid; and chemical destruction of ascorbic acid was accompanied by exactly parallel loss of antiscorbutic activity. Finally, ascorbic acid always had the same degree of activity when isolated from a number of different sources, whether animal or vegetable; and it retained its activity undiminished when chemically repurified or regenerated by different means. Similar arguments have been brought forward by Demole, by Svirbely & Szent-Györgyi, by Tillmans, Hirsch & Vaubel, and by Micheel & Moll, while Dann has reinterpreted Zilva's results (1) in such a way as to show that there is in fact a general correlation between reducing activity and biological potency. Zilva (2), while contesting Dann's interpretation, now agrees that his earlier experiences, which at the time were held to show that the reducing substance was not vitamin C, can be explained on the basis of its reversible oxidation, as first described by Tillmans and his co-workers (Hirst & Zilva). The identity of vitamin C is now all but universally admitted, although it must be mentioned that in the paper just cited we still find so guarded a statement as that the "great disparity in the antiscorbutic activity .... might throw serious doubts on the identity of ascorbic acid with vitamin C." The final proof of course will be the biological tests on a synthetic preparation; and at the time of going to press a brief footnote, "added to the proof," has just appeared in the paper of Reichstein, Grüssner & Oppenauer (1, 2, 3) mentioning, without the details, that their product was in fact active.

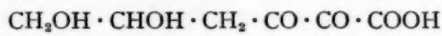
Ascorbic acid has already been used to cure scurvy in man (Schultzer) and in an infant (Parsons).

As to the claims for methyl *nor*narcotine it is necessary to add nothing except that some thirteen further papers have appeared describing negative results.

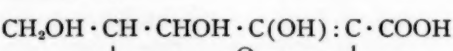
Structural formula of ascorbic acid.—The structure which now finds almost universal acceptance is a later alternative advanced by the Birmingham group of workers, viz.,



The final account of this work, following on a number of preliminary communications (Hirst, Percival & Smith; Hirst; Cox & Hirst; Haworth), is given in a paper by Herbert, Hirst, Percival, Reynolds & Smith. With the addition of fresh experimental facts from various directions, a number of earlier formulations had become untenable, viz., the original formula of Hirst himself,



(Cox, Hirst & Reynolds), the furane carboxylic acid formula of Micheel & Kraft (1, 2),

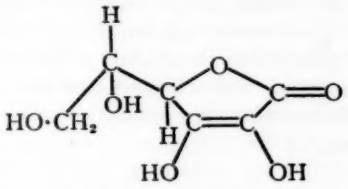


and various rather similar formulae of Karrer (Karrer, Salomon, Schöpp & Morf; Karrer, Schwazenbach & Schöpp). The new formula satisfactorily accommodates all the various observations made by these various workers, and has now been accepted by them all. It also adequately accounts for the actual synthesis of ascorbic acid which had already been accomplished previously by the Swiss workers, Reichstein, Grüssner & Oppenauer (1 and 2), who, however, had at first explained it tentatively on the basis of the then-current formula of Micheel.

There are two specially noteworthy features of the new formula. The first is the presence of a peculiarly reactive grouping, —C(OH)=C(OH)—CO; there are several indications for this, including observations by Euler & Martius on the analogous behaviour of a substance "reductone," CH(OH)=C(OH)·CHO, prepared by heating glucose with alkali. The second of the special

features of the formula is the peculiar lactone structure in place of a true carboxyl group. This was indicated by the fact that the first oxidation product, as pointed out by Haworth and his co-workers, turned out to be not an acid but a lactone.

The main insight into the structure of the molecule was gained by studying its behaviour with oxidising agents (Herbert *et al.*). This showed ascorbic acid to be a derivative of *l*-gulose. The first oxidation product was identified as 2,3-diketo-*l*-gulonic acid. Hence ascorbic acid must be the corresponding reduced derivative of this lactone or 3-keto-*l*-gulonolactone. It only remained to be shown whether ascorbic acid had a furanose or pyranose structure. The nature of the ring system was determined from a study of the methylated derivatives of the acid. This led to the final result that, in solution, ascorbic acid is the enolic form of 3-keto-*l*-gulo-furano-lactone. This formula accounts satisfactorily for the crystallographic properties, a consideration which had led to the rejection of various other alternatives. The X-ray data show that the molecule must be extraordinarily flat, and this is borne out by a diagrammatic representation of the model (I):

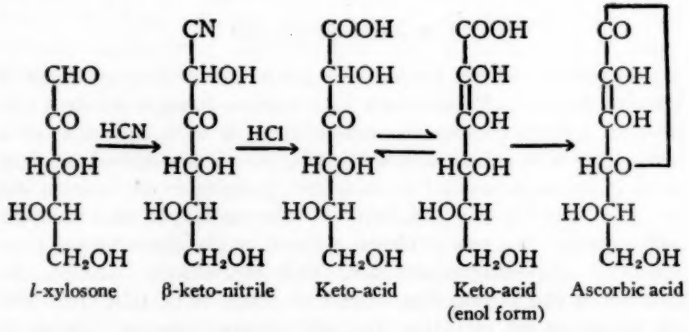


I. Model of ascorbic acid

Independent of the evidence of Hirst and of that of Euler & Martius, Karrer and co-workers have recently brought forward new evidence which supports the lactone formula as against that of a furane ring with a free carboxyl group. Karrer considered this final proof to be really needed as even the synthesis in his opinion did not quite exclude the possibility of the carboxylic acid formula being correct. His new evidence is based on the displacement reaction with alkyl magnesium salt. This conclusively indicates the presence of the lactone ring, since it is found to be split open with the formation of two free hydroxyl groups (Karrer, Schöpp & Zehender). Such accumulated evidence brings the issue to a very satisfactory conclusion.

Synthesis of ascorbic acid.—In their preliminary paper, Reichstein, Grüssner & Oppenauer (2) describe how, when osones are treated with HCN and then hydrolysed, products result which have properties similar to those of ascorbic acid. Starting with *d*-xylosone, the product was found to be identical with the *d*-form (optical antipode of natural or *l*-ascorbic acid). Its identity was proved by separating its acetone derivative, and determining its melting-point, combustion value, iodine titre, etc. In the second paper (3), an abbreviated account of which also appeared in *Nature* (1), the synthesis in larger quantities of both the *l*- and the *d*-forms is described (starting from *l*- and *d*-xylosone respectively), and the former is shown to be identical with natural ascorbic acid; the mixed melting-point showed no depression, and the analytical constants were identical. Finally a brief footnote "added to the proof" (to which we have already alluded) states that tests carried out by Demole showed that the antiscorbutic activity was equal to that of the natural product. (The *d*-form was less active, or possibly inactive, having no measurable effect at four times the level.)

In the preliminary paper describing this synthesis the reaction had been formulated provisionally on the lines of Micheel's formula since that was the only one hitherto suggested in which the oxygen atoms were correctly distributed on the skeleton. With the newer evidence for an "endiol" grouping and a lactone structure, the Birmingham formula is accepted, so that the synthesis is now represented along the following lines:



The synthesis by the nine Birmingham workers, Ault, Baird, Carrington, Haworth, Herbert, Hirst, Percival, Smith & Stacey

(preliminary account by Haworth & Hirst), follows along virtually identical lines. They found that the best conditions, under which a 70 per cent yield of the acid could be prepared from xylosone, were obtained by placing the latter in contact with potassium cyanide and calcium chloride. The reaction involving the addition of hydrogen cyanide proceeds to completion in the course of a few minutes with almost simultaneous hydrolysis to the keto-acid. The conversion of this intermediate product into ascorbic acid proceeds quantitatively in the presence of 8 per cent aqueous hydrochloric acid at 40–50°. "The product is isolated in good yield and with considerable ease as a highly pure crystalline specimen." Its identity with natural ascorbic acid was proved by X-ray examination of the phenylhydrazine derivatives. The steps leading to the preparation of the initial xylosone were: *d*-galactose → *d*-galacturonic acid-diacetone → *d*-galacturonic acid → *l*-galactonolactone → *l*-galactonamide → *l*-lyxose → lyxose-phenylosazone → *l*-lyxosone (*l*-xylosone). At the time of writing, reports of physiological tests have not yet appeared, but the positive results of the Swiss authors may be accepted as sufficient evidence.

Methods of concentration.—Modifications in King's original isolation method have been described by which a yield of 100–150 mg. per litre may be obtained from lemon juice (Waugh, Bessey & King). Baumann & Metzger give details of a simplified procedure by which as much as 16 g. may be isolated from 5 kilograms of iris leaves, or other such plant source. It was the discovery of the large concentrations present in paprika (Hungarian red pepper) which made it possible for Szent-Györgyi to prepare no less than 1 pound of ascorbic acid for the purposes of the international co-operative study of its chemistry, the results of which we have been discussing in the foregoing paragraphs; details of an easy method of preparation from paprika have been published (Svirbely & Szent-Györgyi).

Chemical properties of vitamin C.—Potentiometric studies on ascorbic acid have been reported by a number of workers and some curious anomalies in its behaviour as an oxidation-reduction system brought to light. For details of the measurements and values, the original papers by Green, by Wurmser & Loureiro, by Laki, and by Georgescu must be consulted. Determination of the titration curve by Birch & Harris shows the dissociation constants to be $pk_1 = 4.17$, and $pk_2 = 11.57$.

Attention has been drawn to the interesting fact that the behaviour of ascorbic acid in non-aqueous media may differ fundamen-

tally from that in water, its reducing properties being almost entirely suppressed. It is confirmed also that in an inert atmosphere it is quite stable to moderate heat even in alkaline solution (Herbert *et al.*).

The preparation of a monoacetone derivative (Vargha) has already proved of value in work involving the characterisation and chemical study of the vitamin. This derivative is found to be active when given by mouth but not by injection (Micheel & Moll).

Reduction of ascorbic acid with loss of two hydroxyl groups leads to dehydroascorbic acid, considered to be an inner peroxide (Karrer, Salomon & Schöpp).

An unexpected finding which may lead to important practical developments is that the reducing substance, presumably vitamin C, in milk is apparently extremely sensitive to visible light (Mattick & Kon).

Chemical determination of vitamin C in animal and plant sources. A great stimulus has been given to the study of vitamin C by the development of a chemical test which has led to the discovery that the vitamin is present in high localised concentrations in certain specific animal tissues. The method of test described by Harris & Ray (2) is based on modifications in the use of Tillmans' indicator, 2,6-dichlorophenolindophenol. The essential features are a preliminary extraction process with trichloroacetic acid, followed by titration in acid solution. These conditions are necessary in order to rule out interference by other reducing substances present in many animal tissues, notably glutathione, which would otherwise render the test of little significance. A micro-adaptation of the method has been described by Birch, Harris & Ray (1 and 2), by which titrations may be carried out with ease with an error of less than 3 per cent on amounts of tissue equivalent to no more than 0.03 cc. of orange juice. The test is reasonably specific for vitamin C, but when dealing with unusual types of material it is still necessary to exercise judgment and check results by biological assay. Nevertheless, already in quite a number of cases the test has revealed the presence of vitamin C in unsuspected sites, and the results have been proved accurate by subsequent animal tests. Substances which reduce the indicator in a way similar to vitamin C include (*a*) free cysteine (which may be present in autolysed tissues, but can easily be allowed for, e.g. by a separate Sullivan test), and (*b*) derivatives formed by heating sugars with alkali, and similar substances present in malt extract [Harris

(1)]. The reducing value of tumours [Boyland; Harris (1)] is due partly to vitamin C and partly to a hitherto unrecognised substance, "reducytin," which may be of significance for mitosis and cell growth [Harris (2)].

By the use of this test the writer and his colleagues have been able to prove that large amounts of vitamin C are present in the following tissues: suprarenal cortex (three times as potent as lemon juice) [Harris & Ray (2)]; suprarenal medulla (twice as active as lemon juice) [Harris & Ray (4)]; Jensen rat sarcoma [Harris (2)]; kidney, eye lens, aqueous humour (Birch & Dann). The test has been used also to show that many species, like the dog and rat, can synthesise their own vitamin C when there is none in the diet, while in others like the guinea pig and monkey the development of scurvy is marked by the progressive loss of ascorbic acid from such tissues as the suprarenal [Harris & Ray (2, 3)]. Other writers who have applied the method in various directions include Boyland, for tests on various types of tumour; Svirbely, for animal tissues; Kon, for tests on variability of milk; Eekelen, for invertebrates; Wolff, Eekelen & Emmerie, and Eekelen, Emmerie, Josephy & Wolff for other animal sources; Bennett & Tarbert for lemon juice and orange juice; Euler and his collaborators for various vegetable and animal materials. Eekelen's figures show that after the preliminary reduction with H_2S the titre from certain specimens may be increased as much as two- to tenfold, and a similar conclusion stands out from some of Euler's data.

To sum up some of the applications of the new method of estimating vitamin C chemically, it may be said that it has already been used in such varied ways as for (*a*) controlling works processes, (*b*) guiding isolation operations, (*c*) detecting vitamin C in hitherto unsuspected sites, and (*d*) investigating the effects of storage, exposure, ageing, canning, etc.

It is interesting to note that the staining reaction with silver nitrate, which in the first place actually led Szent-Györgyi to the discovery and isolation of ascorbic acid, is, nevertheless, not given at all by certain tissues rich in the substance, e.g. suprarenal medulla [Harris & Ray (4)]. Gough & Zilva, using this silver test, find that the adrenals, pituitary, and ovary from various species may give a strong reaction, and feeding experiments have confirmed the potency of the ox pituitary.

Distribution.—The presence of high concentrations of vitamin C

in the suprarenal cortex [Harris & Ray (1, 2)], suprarenal medulla [Harris & Ray (4)], and certain other animal tissues (Birch & Dann; Zilva; Wolff, Eekelen & Emmerie; Eekelen, Emmerie, Josephy & Wolff) and in horseradish [Birch, Harris & Ray (1)] and paprika (Szent-Györgyi) has already been alluded to. The corpus luteum, like so many other sources rich in carotene, also contains much vitamin C (Huszák).

It is confirmed that mangoes vary greatly between different varieties (Guha & Chakravorty; Crawford & Perry).

According to the titrations of Kon as well as of Schlemmer, Bleyer & Cahnman, milk is subject to such marked and irregular fluctuations that "the presence of vitamin C in reasonable amounts cannot be guaranteed even in very fresh milk samples of high quality." Exposure to light (even relatively dull light) appears to be a main factor in the inactivation, "which emphasises the value of Nature's method of transference of milk from mother to offspring" (Mattick & Kon). A peculiarly thorough study on milk is that of Bleyer, who has investigated the effects of time after parturition, season, storage, transport, heat, aëration, ultra-violet irradiation, etc. He gives the following relative values: sheep, 3–4; cow, 20–25; goat, 22–28; camel, 40–50; ass, 150. Copper and aëration in commercial pasteurisation are more potent factors for inactivation than heat *per se* (Kieferle & Eisinrich); Sn is less destructive than Cu, and Ni, Cr, and Al least so (Schlemmer & Cahnman).

The statement of Euler & Klussmann (1) that no vitamin C could be detected in urine is not in accord with the experiences of Harris, Ray & Ward, who have followed the day-to-day excretion in patients in relation to the vitamin intake, or with the brief note of Eekelen, Emmerie, Josephy & Wolff.

Unfortunately preservatives which are effective in preventing deterioration in lemon juice also accelerate destruction of the vitamin. It appears that normally in orange juice the vitamin is protected by enzyme systems. Different specimens of lemon juice may vary considerably in potency (Bennett & Tarbert), so that its use as the international standard is unsatisfactory and should be replaced by ascorbic acid [Harris & Ray (3); Key & Morgan].

Physiology of vitamin-C action.—It seems highly probable that the mode of action of vitamin C is related to its unique reducing properties. Harris & Ray (2) have shown that in scurvy the tissues lose their normal content of ascorbic acid and that the lesions which

result in certain sites, e.g. the suprarenals, are associated with this loss. Now it is known that ascorbic acid avidly takes up oxygen, and by its power of *reversible* oxidation we may legitimately suggest that it can function as an oxygen carrier. Ascorbic acid may therefore be regarded as a promoter of oxidative or respiratory systems in the tissues, and it is to be expected that this lack of ascorbic acid in the tissues, which we have demonstrated to occur in scurvy, will result in a diminished oxidative activity. This is in fact confirmed by the measurements of Harrison and of Euler & Klussmann (2), who have shown that tissue slices from scorbutic animals have a low oxygen uptake, which is restored by the addition of ascorbic acid (Harrison). Söderström & Törnblom have shown also that the oxygen consumption of animals is diminished in scurvy.

It may be supposed that it is the peculiar "endiol" system in the ascorbic acid molecular structure which accounts for a specialised physiological action. As to the exact nature of the system in which ascorbic acid participates, significance attaches to the observation that ascorbic acid and glutathione commonly occur in conjunction one with the other (Birch & Dann). These workers have described ascorbic acid and glutathione "as linked factors in one system of oxidation in the animal cell," and they have evidence of a thermolabile agent which acts as catalyst for the dehydrogenation of ascorbic acid. The frequent occurrence of ascorbic acid with the highly unsaturated pigment carotene probably also indicates a common connection in functional action.

Karrer & Zehender state that vitamin C activates the hydrolytic action of cathepsin on gelatin.

The occurrence of very high concentrations of vitamin C in the suprarenal, it is suggested, is not to be taken to indicate that this is the site of its synthesis, nor, on the other hand, that the suprarenal functions as a deposit for reserve stores of the vitamin, comparable for example with the way the liver stores up vitamin A; but rather the explanation is that the vitamin is needed for the purpose of the normal functional activities of the organ [Harris & Ray (4)].

Vitamin-C deficiency in man and experimental animals.—It has been pointed out that subscurvy is very common in parts of Norrbotten (Scandinavia), where no less than 22 per cent of the school children, when examined by Göthlin's test, showed subnormal resistance to capillary haemorrhage (Falk, Gedda & Göthlin). In Sweden at the end of the long winter 10–20 per cent of 500 patients in

a sanatorium showed a subnormal C standard (Forsgren). In North Rhodesia as many as 60 per cent of native labourers (among a group of 10,000) are affected in varying degrees with scurvy (Dry).

Guinea pigs suffering from prolonged partial deficiency of vitamin C frequently develop peptic ulcers, closely resembling the type observed in man, as has been convincingly set out by Smith & McConkey. The possible clinical applications deserve to be borne in mind, as does the advisability of providing fruit juice for patients suffering from the disease.

The presence of vitamin C in the suprarenal [Harris & Ray (1, 2)] suggested the use of vitamin C in Addison's disease, with the result, it is claimed, of clearing up the symptoms of pigmentation after they had failed to respond to treatment with cortical hormone alone (Szüle).

A volume by Hanke fully describes the large-scale tests carried out at Chicago on the action of large doses of orange juice in arresting gingivitis and certain other common dental abnormalities. There seems no disputing the conclusion that the results are statistically significant, as they are striking. Whether the effect is due for certain to vitamin C, of course, cannot be said.

PART II. FAT-SOLUBLE GROUP

VITAMIN A

Limitations as "anti-infective" agent. — Several years ago the writer contested the view that vitamin A could be regarded as a general anti-infective agent of use in the prophylaxis and treatment of all kinds of commonly occurring clinical infections. The grounds for this conclusion (Harris, Innes & Griffith) were: (*a*) that the infections which supervene in vitamin-A deficiency are of a special type, bearing no relation to clinical infections and toxaemias caused by specific pathogenic organisms, but being the secondary result of structural breakdown of membrane tissue and consequent invasion by chance, non-virulent organisms; (*b*) that immunity reactions were not significantly altered by vitamin A; and (*c*) that in this country shortage of vitamin A is not very frequent and where low reserves are found (post-mortem liver analyses) they bear no very close relationship to mortality from many common infectious diseases [Moore (1)]. Additional work during the past year has further supported this view. Thus vitamin-A therapy has been tried, with continued negative results, in a series of large-scale, and statistically significant, clinical experiments, e.g., in the treatment of pneumonia in native mine workers in South Africa [Orenstein; see also Fox (1 and 2)], in prophylaxis against everyday respiratory infections and the like in infants at New York (Hess, Lewis & Barenberg) or school children at Peterhead (Sutherland) and at Königsberg (Erben), in prophylaxis against otitis in scarlet fever (Sutliff, Place & Segool), and in tuberculosis (Gordon & Titherington). Hess opposes Mellanby's view that puerperal sepsis is commonly provoked by vitamin-A deficiency, with the argument that its geographical incidence bears no relationship to vitamin-A shortage, and that where night blindness is in fact prevalent, as in Newfoundland, puerperal sepsis is rare. For a fuller discussion of the clinical significance of vitamin A, a recent address by the writer to the British Medical Association may be consulted (Harris).

In further keeping with the views expressed above on the pathogenesis of the infections in avitaminosis-A are: (*a*) the confirmatory results of Arons & van der Rijst that in experimental animals the keratinisation of the membrane constitutes the primary cause of the infection; (*b*) the clinical observation of Gordon that in fourteen cases of infection in humans in vitamin-A deficiency in Africa the

infection was "from without" and apparently secondary to the lowered protective power of the skin; (*c*) the lack of effect on leucocyte output in hypovitaminous subjects (Hennessy); and (*d*) the diminution in lysozyme in avitaminosis, now confirmed for human subjects (Anderson).

Lesions of avitaminosis-A.—Probably insufficient attention has been paid in the past to lesions of the hard tissues in avitaminosis-A. Simola has recently examined the bones and teeth of vitamin-A–deficient rats, and described osteopathic changes and abnormalities in the odontoblasts and pulp. Wolbach & Howe regard atrophy of the ameloblasts and odontoblasts as the primary effects of vitamin-A deficiency in rats and guinea pigs. The occurrence of tooth hypoplasia has now been noted also in a vitamin-A–deficient infant (Boyle).

Nerve lesions.—It will be recalled that earlier work by Hughes *et al.* and by Mellanby (1) and many others has drawn attention to nervous disorders in vitamin-A–deficient pigs and dogs, chicks, and certain other species. It was the evidence of a condition of subacute combined degeneration in the experimental animal which led Mellanby to suggest that in certain diseases in man in which this same condition is seen, viz. in convulsive ergotism, pellagra, lathyrism, and disseminated sclerosis, vitamin-A deficiency might be one of the principal contributing factors, the other being the presence of a corresponding specific toxin. This theory has as yet been little tested in clinical practice; but Mellanby (2), in a recent lecture, makes the encouraging report that in cases of disseminated sclerosis treated early by dietary means "there has been almost without exception a great improvement even when the symptoms have been extremely severe."

Vitamin A and lathyrism.—The supposition that vitamin A may furnish protection against lathyrism finds some support in a recent important paper by Geiger, Steenbock & Parsons. These workers produced experimental lathyrism in the rat by a diet of flowering sweet pea, and found that adult rats, although not young ones, benefited from the addition of cod liver oil. (Incidentally the sweet pea itself was shown to be a rich source of vitamin A. The toxic factor could be extracted from it by suitable means.)

Vitamin-A deficiency in humans and animals.—Among the records published during the year of endemic avitaminosis-A in different parts of the globe, special reference may be made to an interesting contribution by Mitchell who discusses the conditions at

the Central prison at Uganda; the results of varying changes in the diet, taking place from time to time over a period of years, are clearly apparent in the varying incidence of the deficiency disease. Maxwell has recorded a case of keratomalacia seen in a new-born infant.

An account of ophthalmia in vitamin-A–deficient dogs is given by Stimson & Hedley.

"Hypervitaminosis-A."—Further studies on the effects of excessive doses of vitamin-A concentrates (cf. Takahashi and Harris & Moore) make it evident that bone lesions and damage to kidneys and other soft tissues, as well as exophthalmia and trophic changes in the skin, may be among the more noticeable features [Bomskov & Severs; Bomskov & Seemann; Collazo & Rodriguez; Drigalski; Laubmann; Moore (2)]. Most of the work so far reported is clearly of an interim character.

Concentration.—Using a special high-vacuum distillation apparatus, Carr & Jewell have prepared a concentrate which is appreciably purer than that of Heilbron and collaborators. To judge from their own analytical data (blue value, absorption intensities) they have succeeded in removing some 3–17 per cent of non-active material present in the latter.

Resolution of carotene and vitamin A into various isomeric forms.—Using $Ca(OH)_2$ or CaO as adsorbent it is possible in one single chromatographic operation to bring about the almost complete separation of α-carotene ($SbCl_3$ absorption in chloroform = 542 mμ) from β-carotene (590 mμ) (Karrer, Walker, Schöpp & Morf). By the same process it is claimed that a vitamin-A concentrate can be separated into two fractions, the main constituent being the β-fraction (ultra-violet absorption maximum = 328 mμ, and $SbCl_3$ = 620 mμ) and the minor constituent, the α-fraction (270 mμ and 580 mμ, changing to 620 mμ, respectively). The β-fraction, or vitamin A proper, agrees exactly with the formula $C_{20}H_{30}O$ and the earlier structural formula is confirmed. A similar separation of vitamin A into 2 constituent fractions, using fuller's earth in place of $Ca(OH)_2$, was previously recorded by Eekelen, Emmerie, Julius & Wolff.

An additional, newly discovered form of carotene is the γ-carotene, which is easily adsorbed on Al_2O_3, and shows absorption at a longer wave-length [Kuhn & Brockmann (1)]. From γ-carotene has been further separated a δ-carotene [Winterstein (2)]. β-carotene is twice as active as α- or γ- carotene [Kuhn & Brockmann (2)].

New active carotenoid derivatives.—The belief that carotene occupies a unique position as pro-vitamin A needs to be modified somewhat. β-Hydroxy-carotene, isolated by Kuhn & Brockmann (3) as the unstable first oxidation product of carotene, is as effective as α- or β-carotene, and like it gives rise to vitamin A in the liver. Semi-β-carotenone, which is formed by the opening of one only of the two-ring systems, is active (dose = 5–10 γ), whereas β-carotenone, with the two-ring systems opened, is inactive [Kuhn & Brockmann (4)]. A similar account is given of an active "carotene oxide" (Euler, Karrer & Walker), formed by the addition of oxygen to a double bond in a terminal ring system. Most recently, Winterstein (1) has described a new carotenoid pigment, which like carotene can function as pro-vitamin A; it occurs together with α- and β-carotene in the peel of the fruit, *Gonocaryum pyriforme*. In certain of its properties such as absorption and adsorption it lies intermediate between carotene and lycopin. From a great many papers, too numerous to cite here, we learn that a large number of other carotene derivatives and allied carotenoid substances are inactive.

It has been suggested that, for the hen, xanthophyll has a vitamin-like function (Virgin & Klussmann; Rydbom; MacWalter & Drummond).

Technique of vitamin-A assay.—In Coward's hands, the probable percentage error of an estimation by the growth method, using ten male rats, is +44 or −31 for a one-week test, +28 or −23 for two weeks, +21 or −18 for three weeks, +19 or −16 for four weeks, or +17 or −15 for five weeks. With does the accuracy is slightly less. Coward concludes that it seems hardly worth while continuing a test beyond three weeks, for the sake of the slight additional gain of accuracy. Technical difficulties about the composition of the basal diet are discussed by Bacharach, and by Bacharach & Smith. Culhane refers to a diet on which her rats invariably develop xerophthalmia, which she would regard as a symptom of extreme or complete deficiency.

The vaginal-smear method has been called into question more than once in the past, but recent work all tends to rehabilitate it, provided it is carried out under suitably controlled conditions (Baumann & Steenbock; Aberle).

The inhibiting substance in butter which depresses the $SbCl_3$ index below its true value is found in larger amounts in summer butter, parallel actually with the increase of vitamin A (Booth,

Kon, Dann & Moore). A similar inhibiting substance in cod-liver oil has been isolated by Emmerie. It is now generally recognised that for an accurate quantitative determination a preliminary saponification process is essential, in order to remove such interfering substances. The specificity of the $SbCl_3$ and ultra-violet values, when determined under proper conditions, is well illustrated by the excellent straight-line relationships between them traced by Haines & Drummond, or by the conclusion of Josephy that the two measurements agree with a mean error of less than 5 per cent.

Vitamin-A content of fish livers, and its origin.—The enormous fluctuations in vitamin-A content of halibut liver oil (e.g., from 0.17 per cent to 10 per cent) cannot be readily correlated with the fish's diet; but the principal variable seems to be the age of the fish, old age going with an increased vitamin concentration (Lovern & Sharp; Lovern, Edisbury & Morton). This relation is true also of the codfish (MacPherson), which appears to support the suggestion that the vitamin content of the liver is the result of a gradual accumulation and militates against the theory of a synthesis by the fish itself. MacWalter & Drummond's experiments with young rainbow trout, however, lead them to think that the young fish, before they begin to eat, can synthesise a carotenoid pigment (not carotene) which they convert partly into vitamin A.

A number of Indian fish-liver oils are even more active than that of the cod, although less active than halibut (Chakravorty, Mookerjee & Guha); Pacific Coast ling cod are also very rich (Bailey); so also the waste salmon oil (Eliot *et al.*).

Vitamin-A activity of butter.—It has been shown that the fall in potency of butter in winter in stall-fed cows can be prevented by feeding them on artificially dried grass (Gillam *et al.;* Watson *et al.*). The seasonal variations, and negative effects of chilling and irradiation, are described by Baumann & Steenbock (1). These workers find that carotene accounts for about 15 per cent and vitamin A proper for about 85 per cent of the sum-total vitamin activity (cf. Shrewsbury & Kraybill). Butter from Guernsey and Ayrshire cows is compared by Wilbur *et al.* The Guernsey has about three to four times as much carotene as the Ayrshire, but the amount of vitamin A is the same.

Vitamin A in certain other sources.—The apricot is the richest plant source of vitamin A so far examined (Brockmann; cf. Morgan & Madsen). The activity of Indian mango varies considerably

according to the variety (Guha & Chakravorty). Rapidly growing carcinomas are said to contain more than slowly growing ones (Vogt).

Vitamin D

Vitamin D and clinical rickets.—It is now almost a platitude to say that "no children need have rickets," but it is disconcerting to realise from contemporary estimates [reviewed by Harris (1)] how prevalent the disease still remains in many regions, if admittedly in a less severe form than was formerly common. In 1928 no less than 87 per cent of London elementary school children gave evidence of having had some degree of rickets (Ministry of Health, 1929). One of the latest surveys, from Manchester (Chisholm) mentions that among 1,600 children, 9.2 per cent (in the city area) or 4.6 per cent (in the environs) had pronounced or "definite" rickets.

The final link in the chain between laboratory experiment and clinical application has now been forged by the convincing demonstrations of Spence that purified calciferol itself is invariably efficacious in promoting healing at maximum rate. How conclusive and thoroughly well controlled are Spence's data may be judged from the fact that his "negative control group" actually included several identical twins, with their "opposite numbers" placed in the experimental group. Clinical experience suggests that a suitable prophylactic dose of irradiated ergosterol is 1,500 units and the optimum curative dose 3,000 units: smaller doses may be only partially effective, yet toxic ill effects may occur with as little as 10,000 units or over, so that the necessity for accurate dosing is obvious [Harris (1)]. It has been suggested that the method of measuring vitamin content by the rat technique does not necessarily give an adequate indication of antirachitic potency for humans, since certain sources of the vitamin seem to be relatively more effective than others. Thus, it is stated that the efficacy ratio of irradiated milk, yeast milk, and viosterol is 15:8:1 (Mitchell *et al.;* Hess & Lewis).

The rise in blood phosphatase affords a valuable and very sensitive clinical test for diagnosing rickets and detecting promptly the incidence of the healing process. The average normal figure is 0.25 (range 0.2 to 0.3) as compared with the average for rickets of 0.77 (range 0.41 to 1.29) (Smith).

Vitamin D and caries.—The difficulty experienced in the past in

producing regular symptoms of dental caries in rickety animals seems now to have been overcome by the realisation that a very low blood phosphate is the essential causative factor. For this purpose it is therefore necessary to have a diet extremely deficient in phosphate, this being even more important than actual lack of vitamin D (Klein & McCollum; Agnew, Agnew & Tisdall). The position has now been reached therefore that caries (in experimental animals at any rate) can be produced or prevented at will by an adequate supply of phosphate. The hope of possible clinical applications is encouraged by the report of Grove & Grove that pyorrhoea in humans is correlated with a low blood phosphate. It seems possible even that Hanke's results with orange juice (see page 274 above) may be due to its richness in phosphate. Whether the extraordinarily low incidence of dental troubles in the group of children studied by Sprawson may be due, as he thinks, to their use of raw (unpasteurised) milk and if so whether its peculiar virtue may conceivably lie in its more assimilable phosphate is another possibility to be borne in mind.

Physiology of vitamin-D action.—Two views have been held on the toxicity of excessive doses of irradiated ergosterol. One view was that it was due to toxic by-products and not to the vitamin (Dixon & Hoyle; Underhill; Holtz & Schreiber; Windaus; etc.). The other view was that toxicity was an inherent property of vitamin D itself in excess [Harris & Moore; Harris (2)]. Two arguments were urged for the latter view. The first was that the action of excess was but an intensification of the therapeutic action, so that the symptoms of excess could be predicted in advance, as they were in fact by the writer and subsequently confirmed—viz., a raised blood Ca and P (Harris & Stewart) and resulting excessive calcification at the epiphysis (Harris & Innes) and other sites, and with the anticipated response by the kidneys (Harris & Moore; Harris & Stewart). The second argument was that the degree of toxicity of various products, irradiated by different means, and of products partly or wholly inactivated, went parallel with vitamin potency (Harris & Moore). Dale, Marble & Marks have proved the issue, qualitatively, by showing that pure calciferol is itself toxic; while the writer has shown, quantitatively, that the toxicity of ordinary preparations of irradiated ergosterol is the same as that of their calciferol contents. (It is still true that various artificial partial transformation products may be prepared from calciferol for which the ratio of toxic to thera-

peutic dose may apparently be made to vary according to the treatment given: this may have to do, to some extent at least, with the varying powers of utilisation of these various materials at different levels of dosage.)

The theory that vitamin D functions by stimulating the parathyroid has been advocated by Taylor, Branion & Kay, by Hess *et al.*, and by many others, and is still put forward in numerous current papers. As against this theory Harris & Parks have shown that parathyroid differs fundamentally from vitamin D in that the latter, when given to vitamin-D–deficient dogs, diminishes the faecal output of Ca and/or P, whereas parathyroid has not this typical effect of vitamin D but raises the blood Ca solely by withdrawing it from the bones, thus causing a loss to the body [see also Harris (2)]. This same conclusion that vitamin D works independently of the parathyroid is reached by Dale, Marble & Marks, who find that "complete parathyroidectomy does not prevent or significantly hinder the fatal intoxication produced by large doses of calciferol."

The fact that vitamin D diminishes faecal Ca and/or P may be expressed by saying that it increases "net-absorption" [Harris (2); Harris & Innes], by which term is meant that it causes increased absorption of these elements from the gut or their diminished excretion back into the gut. This conception of "net-absorption" is accepted by Kay & Guyatt since it readily accounts for beryllium rickets, in which the presence of beryllium in the diet would render the phosphate insoluble and hence unabsorbable. The reality of the factor of re-excretion has been proved by Harris & Parks by analysing the contents of the gut for Ca and P, as influenced by the vitamin-D intake, at intervals all along its length. There was clear evidence that material first absorbed in the higher part of the gut was being re-excreted in the lower and was influenced by the vitamin-D intake.

Morgan and co-workers, further, in a comprehensive series of studies on the complementary effects on Ca-P metabolism of the Ca-P intake, the parathyroid hormone, and of vitamin D, reach the general conclusions that their results are in agreement with the views of Harris & Innes that a primary function of vitamin D is to reduce re-excretion, and that its action is not by stimulation of the parathyroids. Pugsley also has confirmed the view that parathyroid in contrast with vitamin D does not diminish faecal Ca or P.

Rickets in various species.—Several dozen papers in all have been

published during the year, discussing the occurrence of rickets in various species, and the most suitable prophylactic measure (e.g., in pigs, rabbits, calves, sheep, chicks, etc.). In the insect kingdom, as exemplified by the cockroach, need for vitamin D however could not be demonstrated (McCay).

Structural formula of vitamin D.—Recent work has radically altered the skeleton of the hitherto-accepted sterol formula. For a full review of the subject see the reports by Rosenheim & King (this volume, p. 87) and by Heilbron, Simpson & Spring.

Are there several forms of vitamin D?—Suggestions multiply that vitamin D may exist in nature in more than one form. It is pointed out that for chicks, irradiated ergosterol is only one-fifteenth or one-twentieth as potent as cod-liver oil fed at the same number of rat units (Bethke, Record & Kennard). There are said to be similar discrepancies between humans and rats, in the relative efficacies of irradiated milk and irradiated ergosterol (*vide supra*). Again, apparent chemical differences are described between the antirachitic factor in butter and cod-liver oil: winter butter loses more than 80 per cent of its antirachitic potency on saponification, in contrast with cod-liver oil and irradiated ergosterol, which are resistant to such treatment (Kon & Booth). It is pertinent further to remark that the vitamin has never yet been actually isolated direct from a natural source and shown to be identical with artificially prepared irradiated ergosterol.

Synthesis of vitamin D in vivo.—Skeptics have sometimes expressed dissatisfaction with the theory that the antirachitic potency of ultra-violet rays can be explained as due to the activation of the ergosterol present near the surface of the skin. The quietus should be given to such criticism by the experimental proof that the active rays are able to penetrate through the epidermis of a day-old rat and then transform an appreciable amount of ergosterol present in solution (Lucas).

Vitamin-D estimation.—The experiences of Coward & Key indicate that the accuracy of the line-test method is such that, by using 20 pairs of rats for a determination, the error will not exceed −33 per cent or +47 per cent 21 times out of 22. Schultzer points out that with the development of rickets in negative controls the width of the rachitic metaphysis increases up to the twelfth day and then subsides somewhat: this change in intensity ("apparent curative effect") may obviously cause erroneous conclusions if conditions are

not all equally standardised. Bacharach *et al.* describe a convenient method for keeping a permanent photographic record of the bone picture.

For estimating vitamin D in concentrated therapeutic solution, an ultra-violet absorption method is simple and accurate (e.g., Fuchs & Beck; Fuchs). An increasing number of workers during the past year have referred to the use of chicks for vitamin-D tests (e.g., Lachat *et al.*).

Vitamin D in various sources.—A large literature deals with the production of milk of a greatly increased antirachitic activity, by feeding cows with irradiated yeast, or irradiated ergosterol (e.g., Krauss & Bethke; Krauss, Bethke & Wilder; Lepsky), and with the result of clinical trials of the product (e.g., Hess, Light, Frey & Gross). Pasteurisation, it is confirmed, does not affect the vitamin-D potency of milk (Krauss *et al.*). Eggs may be made considerably more active by suitable treatment of the hen (Maughan & Maughan; DeVaney *et al.*).

Dates, notwithstanding the fact that they ripen in a tropical sun, are deficient (Lecoq; Morgan). The liver oil of the Norwegian cod (which is taken when the fish is undernourished after spawning) is admitted to be somewhat lower in vitamin A but claimed to be 50–100 per cent richer in vitamin D than that of the Newfoundland cod (which is taken at a season when the fish is in a better nutritional condition) (Poulsson & Ender).

Vitamin E

Bowden & Moore (1) and Morton & Edisbury have described absorption bands of vitamin-E concentrates. It was pointed out by Euler, Hellström & Klussmann that part of the absorption observed, that in the visible, appeared to be due to xanthophyll. This suggestion was confirmed by Bowden & Moore (2), by an actual removal of the xanthophyll. Absorption in the ultra-violet, on the other hand, at 320 and 285 mμ, closely follows the biological activity, in such ways for example as destruction by acetic anhydride and irradiation, and resistance to HCl, hydrogenation, and oxidation. If, as seems likely, these bands turn out to be specific for the vitamin, they should provide an invaluable guide for future work on the isolation of the vitamin.

The development of the histological changes in the testis in vita-

min-E deficiency has been studied very fully by Mason, and compared with the effects of avitaminosis-A. The earliest structural changes are preceded by some fundamental physiological disturbance of an irreversible nature. Histological examination by Zagami & Sindoni, too, confirms that the lesions in the testes are incurable, even after twelve months on a normal diet.

Abortion is very common among sheep in parts of Scotland. In a group of 36 treated with a concentrate containing vitamins E, A, and D, only one aborted, compared with 20 per cent in 650 controls (Dryerre).

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ENERGY FACTORS

Since this is the first review on energy metabolism in the *Annual Review of Biochemistry,* it seemed desirable to include some papers beginning with 1930. Many papers appearing in 1933 were, however, omitted on account of lack of space, but it is hoped to include them in Volume IV.

Specific Dynamic Action (SDA)

Theories of Specific Dynamic Action.—Lusk, long a loyal supporter of Voit's theory, has in his last two papers (1) accepted Rubner's theory not only as regards protein, but also as regards carbohydrates, the SDA of which he previously explained by his own plethora theory. Lusk's last experimental contribution [Dann, Chambers & Lusk (2)] comparing the SDA of meat in thyroidectomized and normal dogs given phlorizin supports Rubner's view that body protein and ingested protein have the same SDA, and that the higher basal metabolism in the diabetic dog is associated with increased protein metabolism. The SDA of protein is the same, whether the glucose derived from the protein is excreted in the diabetic animal or metabolized in the normal animal. Dann & Chambers (3) analyzed liver and muscle of fasted dogs for glycogen, glucose, and lactic acid before and after the ingestion of glucose. A large increase in liver glycogen and a small increase in muscle carbohydrate were found. This was taken as additional evidence for the theory that the SDA of glucose may be associated with intermediary changes in the formation of glycogen, since little or no oxidation of the carbohydrate took place under these conditions, although the extra heat production was about one-half of the normal amount.

Lusk's (4) tribute to Rubner needs to be noted in this connection; as also the fact that Rubner's last paper (5) appeared almost simultaneously with Lusk's last paper, both being historical and reminiscent, as if under the spell of the approaching end; indeed Rubner expressed this feeling to the present reviewer in 1931.

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The researches of Aubel and associates on the SDA of proteins were reviewed in critical detail by Aubel & Schaeffer (6). Reviews of other theories are also included in their communication. Aubel's papers have contributed to the aforecited change in Lusk's ideas on SDA of protein. Adams' brief (7) report was also influential as may be seen by reading the important paper by Chambers & Lusk (8). The observation of Wilhelmj, Bollman & Mann (9) that injected amino acids do not exert any SDA in hepatectomized dogs was probably accepted by Lusk as good experimental evidence against Voit's cell-stimulating theory, and so favored Rubner's theory. The reader must be referred to the review by Aubel & Schaeffer (6) and to Wurmser's monograph (10) for detailed exposition of Aubel's theories. In general, according to these theories, the reactions through which the amino acids go on their path to glucose are endothermic and therefore not realizable spontaneously. Wurmser suggested that in order to make these endothermic reactions possible there must be a coupled oxidation-reduction reaction; coupled, that is, in Ostwald's sense (11). The combustion of glucose constitutes the coupled exothermic reaction. Thus alanine goes through the pyruvic-acid and lactic-acid stages. The coupled reaction involving the oxidation of a part of the formed glucose is represented thus:

$$\text{⅙ } C_6H_{12}O_6 + 4\ H_2O \longrightarrow 4\ CO_2 + 8\ H_2 \quad \ldots -102{,}000 \text{ cal.}$$

$$8\ CH_3 \cdot CO \cdot COOH + 8\ H_2 \rightarrow 8\ CH_3CHOH \cdot COOH \quad \ldots \underline{+160{,}000 \text{ cal.}}$$

$$+\ 58{,}000 \text{ cal.}$$

$$8\ CH_3 \cdot CHOH \cdot COOH \longrightarrow 4\ C_6H_{12}O_6 \quad \ldots -116{,}000 \text{ cal.}$$

$$C_6H_{12}O_6 + 6\ O_2 \longrightarrow 6\ CO_2 + 6\ H_2O \quad \ldots \underline{+667{,}000 \text{ cal.}}$$

$$+561{,}000 \text{ cal.}$$

The total energy liberated by this reaction is

$$58{,}000 + 561{,}000 = 619{,}000 \text{ cal.}$$

or 77,375 cal. per mol of pyruvic acid. So that, per mol of alanine, 30,000 cal. are due to deamination, and 77,000 cal. to the carbon chain, totaling 107,000 cal.

Experimental results on the SDA of alanine, pyruvic acid, and lactic acid, as obtained by Aubel on frogs, by Meyerhof, Lohmann & Meyer on isolated muscles, and by Lusk on a dog, agree, accord-

ing to Aubel & Schaeffer, with the foregoing computations. But Terroine, Bonnet & Zagami (12) could not confirm these results.

From the thermodynamic point of view, the several amino acids might differ in their SDA; and Lusk and co-workers (13) indeed reported that, while phenylalanine, alanine, and glycine have high SDA's, glutamic acid, aspartic acid, and asparagine give negative results. Lusk and co-workers refer with approval, as possibly explaining their results, to calculations by Adams (7), who with the aid of the third law of thermodynamics showed that the free-energy increment, ΔF, is negative for the conversion of glutamic acid into glucose; but that at least 0.871 Cal. are needed to drive the reaction for 1 gm. of glycine into glucose and urea, and 0.337 Cal. to drive the reaction for 1 gm. of alanine into sugar and urea.

Borsook & Winegarden (14) criticized the computations of Adams on the ground that the specific heat data at present available are too scanty to permit reliable estimations of the entropies of alanine and of glycine. Accordingly, calculations of glycine to glucose and urea, although the standard free energies of glucose and urea are known, are at present uncertain. However, Borsook & Winegarden confirmed Adams' computations for alanine and glycine, making the estimations by means of the first law of thermodynamics; that is, they agreed that the conversion of glycine and alanine into glucose and urea are endothermic processes requiring the driving force of another exothermic reaction, and if the relationship between the endothermic and exothermic processes is not perfectly reversible, then the SDA will be the difference between the amount of energy produced and the amount used in the endothermic reaction. But there are no data for estimating the degree of reversibility of such a reaction in the body. If a 25 per cent efficiency is assumed, then the SDA per mol of glycine would be approximately 200,000 calories. On the same basis, the SDA of alanine should be one-third that of glycine; yet the experiments of Lusk, of Wilhelmj & Bollman (15), and Wilhelmj & Mann (16) all indicate that the SDA is the same for glycine and alanine.

As regards glutamic acid, Borsook & Winegarden argue that the reaction is exothermic; the value of ΔH is equivalent to the combustion of one-fourth mol of glucose. But there is no obvious justification for assuming, as did Lusk and Aubel, that because this reaction is exothermic it is not responsible for any SDA. Rather the reverse; it seems that this is one reaction of the three which would cer-

tainly exert an SDA; for even in the case postulated for glycine and alanine the SDA is due to exothermic processes. Moreover, gliadin, which contains over 40 per cent of glutamic acid, exerts a large SDA.

Returning to Lusk's findings that glutamic acid, aspartic acid, and asparagine give no SDA, Terroine, Bonnet & Zagami (12) found on the contrary that, per gram of nitrogen administered, the SDA of glycine, alanine, aspartic acid, glutamic acid, valine, leucine, cystine, and lysine are approximately the same, namely 8.4 Cal.; for tyrosine and phenylalanine, 9.2 Cal.; for tryptophane and histidine, 10 Cal. Terroine & Bonnet were much impressed by this constancy and concluded that the extra heat production in a thermally neutral environment resulting from the administrations of different amino acids is constant, namely, 8.4 Cal. per gram of nitrogen metabolized (17).

Lundsgaard (18) reached still more surprising results. He found a nearly constant extra heat production, namely about 8 Cal. per gram of nitrogen administered, during the five hours following the administration, not only of glycine, alanine, glutamic acid, aspartic acid, and tyrosine, but also for ammonium salts such as ammonium chloride. This author found no increase in oxygen consumption after the administration of sodium salts of glycollic acid and lactic acid, or the hydroxy acids corresponding to glycine and alanine. But the ammonium salts of these acids increased the oxygen consumption in a manner very similar to that of amino acids. The action of ammonium was found to be practically the same as that of alanine. It should be noted that the heat increment of 8 Cal. found by Lundsgaard is, within the limits of experimental error, the same as that found by Terroine & Bonnet. It should be noted further that as far back as 1916 Grafe reported (19) that ammonium chloride and acetamide exert a large specific dynamic effect.

Borsook & Winegarden (20), moreover, found that urea exerts a large SDA, namely, 6 to 11 Cal. per gram of nitrogen excreted; the average value is of the order of 9 Cal. per gram of nitrogen, which, within the limits of experimental error, is the same as the value reported by Terroine & Bonnet, and by Lundsgaard. Shortly before this, Lublin (21) observed a rise in metabolism of from 8 to 12 per cent in similar experiments. The experiments of Lusk and Grafe, which led them to conclude that ingestion of urea does not increase metabolism, are well known. Borsook & Winegarden criticized their conclusions. Brody & Procter (22), working over the classic data by Williams, Riche & Lusk (23), found that the ratio of SDA to extra

urinary nitrogen excretion after a heavy meal of meat declines rapidly after feeding, and that the average ratio for a period of 21 hours after feeding is of the order of 11 Cal. per gram of urinary nitrogen.

Considering the difference in technique and difference in animal species employed by the several investigators, the range in values of SDA per gram of nitrogen metabolized is relatively narrow, namely, 6 to 11 Cal. per gram of nitrogen, and apparently independent of the nature of the nitrogen source.

There are, to be sure, a number of discordant notes from other laboratories. Thus Zummo (24), in Lombroso's laboratory, found that glycine when given in 5-gram doses by mouth to pigeons exerts no SDA. Stassi (25) found that alanine and glycine have no SDA in normal pigeons, and the response is not altered by previous fasting. Barbato (26) reported that alanine and glycine given to pigeons do not increase the oxygen consumption, while aspartic and glutamic acids do. With aspartic acid, the oxygen consumption reaches a maximum in 2 hours, remains constant for about 3 hours, then returns to normal. A longer period is required to return to normal in the case of glutamic acid. Zummo (27) found that ammonium citrate exerts no SDA. Somewhat later Zummo (28) published data which led him to conclude that the SDA of nitrogenous substances does not depend on the conversion of ammonia to urea or uric acid.

An apparent exception to the view that protein exerts an SDA was reported by Mark (29) who states that muscle proteins have unique heat-stimulating properties and that liver protein exercises an insignificant SDA, or may even depress heat production. The proteins from the spleen, kidney, and thymus have reduced specific dynamic effects. Schlumm & Brechmann (30), however, did not find concordant results on the differences between the SDA of liver and muscle nitrogen, although in most cases calf's liver appeared to have a lower SDA than muscle.

The fact that urea and ammonium chloride exert an SDA suggests the possibility of other factors entering the phenomenon than those assumed by Rubner and his modern followers. May not the Zuntz theory (31) explain it? According to this theory, the SDA represents the energy expense of digestion, absorption, excretion, and secretion. The term employed by Zuntz, namely, *Darmarbeit,* or *Verdauungsarbeit,* is rather too narrow to include all these processes, and so leads to misunderstandings.

Zuntz's theory was apparently discredited by Rubner, Benedict, Lusk, and others. The experiments of these investigators consisted largely in feeding such substances as bones, Glauber's salts, agar-agar, popcorn, and other roughages and irritants, or Liebig's extract of beef. They found no appreciable increase in heat production following this type of feeding, and they consequently concluded that Zuntz's theory of *Verdauungsarbeit* cannot be true. But in this conclusion they seem to have overlooked the fact that this term as used broadly by Zuntz was intended to include not only the mechanical work of peristalsis, but also the physico-chemical work of secretion, absorption, and excretion; and, of course, there is relatively little physico-chemical work connected with the ingestion of bones, agar, or even of cathartics or Liebig's extract of beef.

Borsook & Winegarden (32) in a series of three papers present a good case in favor of the view that the energy expended by the kidney in its secretory and excretory processes contributes largely (25–60 per cent) to the SDA. This, of course, would be substantiating Zuntz's theory of SDA, although perhaps not in the anticipated manner. The remainder of the SDA of protein or amino acids is due to the metabolism of nitrogen and carbon. In the first paper they presented an analysis by means of the second law of thermodynamics of the work of the kidney in the excretion of urine. They concluded that the work performed by the normal kidney in man in the excretion of urine is of the order of magnitude of 0.7 gram-calories per cc. of urine; or 70 gram-calories per gram of nitrogen excreted. The second paper based largely on their urea-ingestion experiments concluded that the energy expended by the kidney in man in the production of urine is 6 to 11 Cal. per gram of nitrogen excreted; therefore, the efficiency of the kidney is probably not greater than 1 to 2 per cent. Lundsgaard's paper, which appeared after Borsook's papers, seems to substantiate these conclusions. The third paper is devoted to a critical analysis of published data, largely that of Lusk and his pupils, and to drawing the following conclusions from them: (*a*) there is a close correlation between the SDA and the extra nitrogen excretion; (*b*) the evidence does not support the view that SDA is necessarily due to conversion of the deaminized fractions into glucose; (*c*) 25 to 60 per cent of the SDA is due to the work of renal excretion, and the remainder to the metabolism of nitrogen and carbon; (*d*) the hypothesis in *c* above, that the SDA of protein is due to at least two distinct processes, which do not proceed at similar rates, provides an

explanation for some hitherto anomalous phenomena in the SDA of protein, as for example the apparent differences in the SDA per mol of different monoamino acids, or the apparent decrease of SDA after hypophysectomy or pituitary deficiency.

The increased oxygen consumption of the kidneys associated with the excretion of nitrogenous residues is by no means primarily a matter of energy expenditure for the work of secretion or excretion of the nitrogenous residues. Krebs (33) found that rat kidney tissue *in vitro* deaminizes amino acids (to form NH_3) far more rapidly than rat liver or any other organ (the rate of formation of NH_3 being as high as 0.0038 mg. per hour per mg. of dry kidney substance), and that oxygen is essential for this reaction, as indicated by the equation:

$$R \cdot CH\ (NH_2) \cdot COOH + O \rightarrow R \cdot CO \cdot COOH + NH_3$$

Alanine, aspartic acid, glutamic acid, and cystine caused marked increases in oxygen consumption. The corresponding keto acids caused similar increases. Incidentally, this author (34) reported identification of α-keto acids as intermediate products in the oxidative deamination of amino acids by kidney tissues, but not with the dibasic acids, aspartic and glutamic. The reaction may be stopped at the keto stage by poisoning the tissue cells with 0.001M As_2O_3, in which case aspartic acid and glutamic acid yield the same type of products as the monobasic amino acids.

Glaser, Laszlo & Schürmeyer (35) found a linear relationship between the amount of blood flow through the kidney and its oxygen consumption. As the energy consumption of the kidney, determined by indirect calorimetry, far exceeds all the known work the organ has to do in the formation and elaboration of urine, they suggest that some endothermic reaction hitherto unsuspected must be carried out by the kidney, and possibly this is the formation of ammonia.

Borsook & Keighley (36) investigated the synthesis of urea from ammonia and carbon dioxide in the presence of living rat-liver slices in Ringer's solution, finding a definite increase in oxygen consumption. It appeared that one additional molecule of oxygen is used for every molecule of urea synthesized, and that the total energy exchange is governed by the stoichimetrical rather than by the energy requirements of the reaction in which the free energy, ΔF, is positive. Similar values were obtained with both glucose and *dl*-lactate as fuel, and with or without ornithine. Comparison of the rates of synthesis

of urea suggests that the fuel in this reaction is lactate or some product derived from it. They concluded that "it seems unlikely that not more than a fraction of the SDA of protein can be accounted for by the superfluous energy released in the synthesis of urea from ammonia." In a somewhat later paper (36) again using rat-liver slices, an increased oxygen consumption was found to accompany the synthesis of urea from $(NH_4)_2 CO_3$. The authors concluded that this process is probably responsible for not over 20 per cent of the SDA of protein.

Aubel (37) found that aqueous extracts of hashed dog liver or kidney oxidized alanine to pyruvic acid, but did not oxidize sodium lactate. Under the same conditions extracts of muscle, spleen, pancreas, and lung had no action.

As a matter of fact, at least one report favors the liver rather than the kidney as the principal seat of the SDA reactions. Dock (38) found no increase in oxygen consumption by the rat kidneys on a high nitrogen diet; 85 per cent of the extra heat production of the rats (chloretone-anesthetized) on high-protein diets was liberated in the abdominal viscera, probably chiefly in the liver. His method consisted in measuring the oxygen consumption of two groups of rats, one fed casein and vitamins, and the other starch and vitamins before and after ligation of (*a*) the abdominal aorta below the renal artery, (*b*) the renal artery, (*c*) the splanchnic arteries. The oxygen consumption of the abdominal viscera was found to increase by 191 per cent during the high-protein feeding, while that of the hind quarters increased by 8 per cent. The total oxygen consumption was increased by 35 per cent. Dock concluded that the SDA is due to the liver forming glucose from amino acids.

Note was made of the suggestion that the SDA of protein is due to at least two distinct processes (metabolism of nitrogen and carbon; work by kidney) which do not proceed at similar rates. Ralli, Canzanelli & Rapport (39) also emphasized that the urinary nitrogen excretion does not accurately represent actual protein metabolism of the period, because of the delay in urea formation and excretion. In cases of apparently lowered SDA as in hypophyseal deficiency, there appears to be no real reduction in SDA per gram of nitrogen metabolized but only a slowing of the rate of metabolism. The older literature on the influence of endocrine deficiencies on metabolism is reviewed by Lusk (13). The recent literature confirms the view that the SDA is not influenced quantitatively in hypophyseal de-

ficiency. Artundo (40) measured the SDA on six hypophysectomized dogs and six controls. Hypophysectomy reduced the average metabolism by 15 per cent. The absolute increase of metabolism after feeding a protein meal was the same for both groups but the initial level was lower for the hypophysectomized animals. Johnston (41), after investigating the SDA in six humans with definite abnormalities of the pituitary body, concluded that there is no evidence showing any dependence of the SDA of protein upon pituitary activity, as the SDA response was quantitatively normal in every case. Fulton & Cushing (42) examined the SDA in 21 cases of acromegaly (BMR, —11.5 per cent), 32 of hypopituitarism (BMR, —19 per cent) and 29 with para-hypophyseal or other intracranial tumors and found that these disorders have no effect on the SDA of proteins. The SDA of beef was the same in acromegaly (19 per cent) and hypopituitarism (23 per cent), but the maximum effect appeared later in hypopituitarism. Daggs & Eaton (43) found that the SDA of beef heart fed to hypophysectomized dogs was not below but rather slightly above normal in these dogs when calculated as calories increase over basal. Mazzocco (44) compared the SDA of meat in (*a*) normal dogs, (*b*) hypophysectomized dogs, and (*c*) dogs with the pars tuberalis cauterized. No difference was found between (*a*) and (*b*), but the SDA was slightly higher in (*c*), especially after a 3-day fast. Braier (45) found that the essential metabolic difference between hypophysectomized and normal dogs is that in the former there is a delayed elimination of urinary nitrogen due to a given meal of meat. Udaondo & Goñalons (46) reported that it is very difficult to evaluate the SDA in hypothyroid or genital obesity, since the SDA depends on the functional state of endocrine organs, and on the time relations of digestion or delayed effects of previous diets.

Nor is the state of nutrition, or obesity, an influential factor on SDA (47, 48, 49).

As regards the SDA of carbohydrates, mention was already made of the fact that Lusk, Chambers, and associates explained it to be due to intermediate changes, agreeing in principle with Rubner's theory.

Bonnet (17) found that glucose when injected into the blood stream even in relatively massive doses does not exert any SDA, while when given by mouth the heat production is increased by about 4 per cent. He considered this as substantiating Rubner's theory. But

it may as well be considered as substantiating Zuntz's theory as representing the cost of *Verdauungsarbeit*.

Carpenter & Fox (50) reported that, while a part of the SDA of glucose appears to be used for muscular work, none of the SDA of fructose is thus used. It is known from Anderson & Lusk (51) that the SDA of carbohydrates may in part be used for work, and they explained it at that time by Lusk's plethora theory, but what is the explanation of the difference in behavior in this respect between glucose and fructose? According to Rapport (52) the SDA of glucose and fat are abolished during muscular exercise, indicating that it can be utilized as free energy in muscular work. But the SDA of tyrosine and the energy due to the calorigenic action of adrenaline were not utilized in muscular work.

Lusk's plethora-, or Krummacher's mass-action theory as it relates to fats, has not been challenged. Gregg (53) found that the SDA of lecithin given *per os* to dogs is below that for isocaloric amounts of neutral fat, and when injected intravenously in amounts up to 1 gm. per kilogram it has no calorigenic effect. The results do not support the view that lecithin is concerned in the intermediary metabolism of fat, nor that it is an available source of material for oxidation.

Other papers on SDA.—A notable series of papers appeared by Carpenter & Fox on the influence of ingestion of carbohydrates in humans on the gaseous exchange. The introductory paper (54) presented data on the influence of ingestion of water at 37° C. by a person in post-absorptive condition. If the water ingested was less than 250 cc., the course of carbohydrate metabolism and the heat production changed little, if at all. The ingestion of 250 cc. of water was followed by a slight, but definite increase in the metabolism of carbohydrate for a period of one hour (average 4 per cent); and with 500 cc. there was an increase (42 per cent) lasting two hours. The heat production was slightly increased (2 per cent) for 1½ hours in experiments with 200 cc. of water, for two hours (1 per cent) with 250 cc. of water, and for two hours (7 per cent) with 500 cc. of water at 37° C. The authors suggest that the transfer of fluid in the body involves expenditure of energy. The authors cite Lublin, who attributed the higher metabolism following water ingestion to renal work, and Grollman, who attributed it to general stimulation of body cells due to dilution of the internal environment of the cells by the ingested fluid. The second paper (55) reported on the

respiratory exchange after the ingestion of dextrose in amounts between 5 and 104 grams, in water at 37° C. The increase in heat production was practically 1 Cal. for each additional gram from 5 to 25 grams. Above 25 grams, the heat production rose more rapidly in proportion to the additional amounts given. The maximum R.Q. was observed in the third half-hour. Ingestion of 10 grams or over was followed by an increase in R.Q. The increase in carbohydrate metabolism above the basal for three hours represented on the average from 17 to 31 per cent of the amount of dextrose ingested in quantities of 10 to 36 grams. The energy of the SDA varied from 4 to 6 per cent of the energy of the dextrose when 5 to 36 grams were taken, and 10 per cent when 52 grams were taken. The third paper (56) is an extension of the preceding research to include levulose. The SDA of levulose varied from 2 per cent with 21 grams of levulose to 12 per cent with 104 grams. The maximum R.Q. was found mostly during the second half-hour after levulose, and with larger quantities the increases in the quotients were larger and continued later than with smaller quantities. The increase in apparent catabolism of carbohydrate represented 30 to 34 per cent of the amount of levulose given to the subject, with the exception of 14 per cent in the experiments with 5 grams of levulose and 53 per cent in the experiments with 15.5 grams. Increases in heat production were found in all groups, which varied from 0.5 Cal. in one hour after ingestion of 5 grams of levulose to 46 Cal. in four hours after 104 grams. The minimum increase in proportion to the amount given was 1.5 Cal. in two hours after 21 grams. The authors suggest that the reaction after levulose is one of conversion of carbohydrate into fat even when the R.Q. does not rise above unity.

In the case of galactose, the SDA varied from 3 to 8 per cent of the heat of combustion of this sugar [Carpenter & Lee (57)] when the amounts ingested varied from 5 to 40 grams. The rise in apparent carbohydrate combustion varied from 1.1 to 12.8 grams, which represented from 13 to 32 per cent of the amount ingested. Galactose resembles glucose in respect to heat production but it behaves more nearly like fructose with regard to its R.Q. The increase in the R.Q. varied from 0.04 with 5 grams to 0.17 with 40 grams. There appeared to be some sex differences in the respiratory exchange under the influence of galactose (58). The outstanding sex differences were: (*a*) greater maximum rise in the R.Q. in women; (*b*) greater initial increase in heat production after 20-gram doses in

women; (*c*) greater increases in carbohydrate combustion and heat production in the men after 30-gram doses; (*d*) greater individual variations in all the factors with women.

When 20 grams each of glucose and fructose were taken together, the increase in heat production equaled the sum of the effects of these two sugars taken separately. But when galactose was combined with either of these sugars there was a greater increase in heat production than would be expected from the summation of the effects of galactose and any other sugar taken separately. The authors suggest (59) that this increase may be due to (*a*) more unabsorbed galactose in the intestine when another sugar is present; (*b*) greater formation of lactic acid in the intermediate metabolism of galactose; (*c*) smaller formation of glycogen where other glycogen formers are available.

Wesson (60) measured the metabolic rate and R.Q. of rats following the ingestion of dextrin. Before the determinations, the animals were kept in the dark and at 28° C. for three days. Two methods of determination were employed: (*a*) a bell jar from which expired air was collected at intervals, and (*b*) a special closed circuit apparatus. At the commencement of each experiment, which ran up to 44 hours, each rat was given a predetermined test meal of dextrin. By the 14th hour the metabolic rate reached a basal value. The R.Q. decreased rapidly from 0.91 for the second hour to 0.744 at the 16th hour, then slowly, with several slight variations, to 0.738 at the 43d hour (R.Q. not corrected for protein metabolism).

Lombroso (61) found that 3 to 4 grams of sucrose given in water by mouth had no appreciable effect on the gas exchange of pigeons or rats. In dogs, sucrose given orally or glucose given intraperitoneally raised the metabolism by up to 30 per cent. Frisco (62) found that in most cases administration of olive oil causes a definite and often a considerable rise in oxygen consumption in pigeons at neutral temperatures.

May & Meyer-Heine (63) studied the increase in the metabolic rate in normal subjects 1½ hours after a test meal of 200 grams of veal, 100 grams of bread, and 100 cc. of water. The increase is at least 10 per cent, and, on the average, 20 per cent. Urea excretion during the same period runs parallel to the SDA. Kaplan (64) found that 3 meals of 1,800 Cal. made up of combinations of bread, meat, bacon, buckwheat porridge, sugar, and tea caused in two humans increases in metabolism of 19–38 per cent. This increased metabolism could not be used for work. Meals with half-portions were followed

by increases of 13 to 22 per cent. Meyer (65) found that the ingestion of 500 grams of raw lean beef by a 10-kilo dog at rest was followed by an increased heat production, over basal, of 47 Cal. After an 8-hour run; the same amount of meat resulted in an increase of 72 per cent over that following rest. The author suggested that the greater SDA after work is due to the extra chemical work which the organism performs in order to transform the ingested protein into materials suitable for the metabolism of muscles. Castex & Schteingart (66) injected 0.2–0.4 grams NaCl subcutaneously with a resulting rise in BMR of about 11 per cent (range 4–19). The peak was reached in one-half hour; the metabolic rate returned to normal in an hour.

Net Energy and Feeding Standards

The problem of formulating proper feeding standards is one of the two most important in the practical enterprise of efficient production of animals and animal products. Its importance in human nutrition under conditions of stress is well known. Some of the following remarks will indicate the difficulties of this problem as they relate to the energy factor. We shall review the literature as related to cattle, and make but brief mention of the few papers concerned with humans.

Before formulating a rigorously quantitative feeding standard for energy one must not only know the physiological needs of the animals for energy, but also the net or available energy of the food. To obtain the net energy one must deduct from the gross energy of the food the energy of (*a*) feces; (*b*) urine; (*c*) fermentation gases; (*d*) energy of SDA; (*e*) the energy expense of converting the available nutrients to body substance or other desired products such as milk, eggs, wool, etc. The energy of *a* is easily determined and thus the digestible nutrients are easily evaluated; *b* is likewise determined without much difficulty so that the metabolizable energy or the physiological fuel value of the food can be evaluated easily. On the other hand, on production rations, it is very difficult if not practically impossible to determine *d* and *e* because: first, it is not possible to differentiate between *d* and *e*, when the animal is fed on a productive ration; second, under practical feeding conditions the plane of nutrition affects particularly the "waste heat" of the SDA; third, the foods have a mutual influence on each other so that the net energy varies with the food mixture; fourth, in the case of farm animals it

takes 2 to 3 days to evaluate the SDA of a given food, during which time the animal spends more or less energy for muscular movements, which energy expense cannot be differentiated from *d* and *e* above; finally, should the environment temperature vary, the heat production would perhaps also vary so that *d* and *e* would be further confused. The discussion leads to the conclusion that it is very difficult indeed to evaluate the net energies of foodstuffs, and it is consequently very difficult to formulate scientific feeding standards. These uncertainties have become clear only in the past few years as indicated in the following review.

Influence of the plane of nutrition on the net-energy of foods.—Forbes and associates (67) initiated this work on beef steers followed by Wiegner & Ghoneim (68) on a rabbit and by Mitchell *et al.* (69) on a beef steer.

We are indebted to Forbes *et al.* not only for initiating these experiments but also for most excellent data. In two phenomenally concordant series of studies on energy metabolism in relation to the plane of nutrition Forbes *et al.* determined energy balances with four steers at seven planes of nutrition (fasting; ½-maintenance; 1-maintenance; 1½-maintenance; 2-maintenance; 2½-maintenance; 3-maintenance). They found that with the heat production of the fourth day of fast as the basal value (when the R.Q. for fat is attained) the heat production increased slowly between fasting and maintenance, and much more rapidly above maintenance, but with a decreased rate of increase between the higher points of observation. The curve of heat production in relation to the plane of nutrition was found, therefore, to be a reversed or S-curve. The diminished rate of increase in heat production between the higher points of observation was interpreted as caused by the diminished metabolizability of the rations at these planes of intake.

Wiegner's chief interest appeared to be to formulate a quantitative generalization of energy utilization. Wiegner maintained that the theory that the net energy of food is directly proportional to the quantity of metabolizable energy is unreasonable since an increase in production does not continue indefinitely. In other words there is an upper limit beyond which further food intake cannot be utilized productively. According to Wiegner & Ghoneim, therefore, the net energy of a foodstuff does not vary linearly with the food ingested (as was generally assumed until the appearance of the paper by Forbes *et al.*) but the successive increments of net energy decline

with successive increments of food consumption in accordance with the differential equation: $\frac{dA}{dF} = K\,(H - A)$, in which A is the net energy of the foodstuff; F is the metabolizable energy of the food; H is the maximum net energy of the food intake; K is the efficiency coefficient; or the integrated form $ln\,\frac{H}{H - A} = KF$. Wiegner & Ghoneim found their equation to represent the data of Forbes's steers as well as their own on the rabbit. Grünigen (70), in a dissertation prepared under Wiegner's direction, extended Wiegner & Ghoneim's mathematical discussions.

Shortly after the appearance of Wiegner & Ghoneim's papers, Mitchell, Hamilton, and associates published a similar set of data on a very large steer (613 kilos as compared to Forbes's 433-kilo steers). The ration fed to this steer likewise differed considerably from the ration fed to Forbes's steers, Mitchell's steers receiving half as much corn. Unlike Forbes, Mitchell & Hamilton believed that from the maintenance level upward, that is within the practical range of feeding, the heat production is a linear function of the dry matter consumed, and that also "the net energy of a ration bears a linear relation to the amount of dry matter consumed."

Mitchell & Hamilton emphasized "that the lowest level of feeding was associated with the most complete digestibility of all nutrients." They found that from the highest to the lowest level of nutrition the metabolizable energy of the ration per kilogram of dry matter increased from 2661 to 3094 Cal.; the percentage utilizability increased from 60.6 to 70.1. But when the metabolizable energy is computed per kilogram of digestible nutrients all effects of the level of feeding disappear. The net energy increased from 1,842 to 2,957 Cal. and the average daily R.Q. decreased from 1.13 to 0.88. The percentage availability of the metabolizable energy increased from 69.3 at the highest level through 73.8, 75.2, 77.7, and up to 95.6. This rise is due to increased digestibility and decreased specific dynamic action.

The tremendous differences in size of the animals as also the differences in rations and differences in methods of presenting results employed by these three groups of investigators made it difficult for the reader to compare the three sets of conclusions. Brody & Procter (22) attempted a graphic and mathematical analysis of the three sets of data with the object of expressing all results in comparable units, and in general to harmonize them. This analysis led to the following

conclusions: (*a*) the digestibility of typical rations in these animals is very low compared to digestibilities of diets in humans as quoted by Sherman (71). About 28 per cent of the gross ingested energy in these animals is lost in the form of feces. These losses increase with the increase in the plane of nutrition. The range of increase in the losses with the increase in the plane of nutrition is not, however, very great (range 5 to 10 per cent); (*b*) the methane losses in ruminants are quite considerable (about 9 per cent of the gross energy); but the range in losses from the lowest to the highest plane of nutrition is not very great (range about 3 per cent); (*c*) the energy loss in urine is rather small (about 5 per cent of the gross energy), and the range in losses from the lowest to the highest plane is quite small (about 1.5 per cent); (*d*) the energy losses due to specific dynamic action (heat increment of feeding) vary enormously with the plane of nutrition (ranging from about 3 per cent of the gross energy at about one-half maintenance to about 20 per cent of the gross energy at maximum feed intake). *The net energy per unit of food intake thus declines with increasing plane of nutrition largely on account of the increase in the heat increment of feeding;* (*e*) it is shown that the net energy, Q_n, changes with the intake of gross energy, Q_g, in a given animal according to the equation

$$Q_n = A\,(1 - e^{-kQ_g})$$

in which A is the maximum net-energy intake capacity; k is the relative (or when multiplied by 100, percentage) change of Q_n with changing intake of Q_g; e is the base of the natural system of logarithms. In this equation, the numerical constants vary with the size of the animal. In order to eliminate the size factor it is necessary, first, to divide A by weight, area, or some other equalizing size factor, the nature of which has not yet been determined on account of paucity of data; second, to plot Q_n not against the amounts of food consumed, but against the plane of nutrition (1-maintenance, 2-maintenance, etc.). Since the former problem has not yet been solved the ratio of net to digestible energy (Q_n/Q_d) was plotted against the plane of nutrition (Q_p). The steer and rabbit data in this case nearly coincided and the equation for the curve was then found to be

$$Q_n/Q_d = Ae^{-Q_p} + 54$$

indicating that the net energy of the given rations cannot decline below 54 per cent of the digestible energy.

It is necessary to emphasize that the extra heat following feeding represents not only SDA but also the energy of unavoidable muscular movements of the animals during the 3-day test, the cost of conversion of nutrients to body fat, etc., and possible influences of fluctuating environmental temperature.

The mutual influence of foods on their net energy.—It is generally known that the "biological value" of a food (referring to the protein components of the food mixture) is influenced by the other foods with which it is mixed in compounding the ration. Forbes suggested (72) that the net energy of a food is likewise influenced by the other components in the ration. Thus according to Forbes (73) the apparent metabolizable energy of corn meal fed on a production level to steers varied with the kind of roughage with which it was fed from 2,946 to 3,339 Cal. per kilogram of dry matter in one steer, and from 2,944 to 3,387 in another. The apparent net energy differed between 1,645 and 2,518 Cal. per kilogram. Forbes (72) has formulated what he termed "the law of maximal normal nutritive value," according to which a food expresses its normal and most characteristic nutritive value for a given kind of animal under specified conditions only as a part of a ration which is qualitatively complete and quantitatively sufficient for the existing conditions. The practical conclusion follows that the net energy values of individual foodstuffs are fundamentally variable, and that "the most nearly logical, single, conventional measure of whole nutritive value is the net energy of the nutritively complete ration."

It is a difficult matter for a reviewer to attempt to evaluate critically the arguments for and against the use for practical purposes of "net energy," "metabolizable energy," or "digestible energy." In some cases the energy lost through SDA is as much of a loss as the energy losses in urine or feces, and should logically be deducted from the gross or digestible or metabolizable energy of the food. On the other hand the experimental or computational errors in evaluating the SDA are considerably greater than for evaluating the digestible and metabolizable energy already cited.

Feeding standards and energy content of foods.—We shall discuss briefly the feeding standards proposed for maintenance and milk production in dairy cattle, and note the few contributions to this subject as they relate to humans.

Cattle.—Kriss (74) reviewed critically, and compared, the feeding standards for dairy cattle as elaborated by Forbes, Kriss, and asso-

ciates with those previously published. The accompanying tables are a condensed summary of the comparisons as they relate to the energy aspect of the standards.

FEEDING STANDARDS FOR DAIRY CATTLE

Maintenance Standards per 1,000 Pounds Live Weight per Day

Author	Net Energy "for Fattening," Calories	Digestible Nutrients, Pounds
Kellner	6,200	6.67
Armsby	6,000	6.46
Mollgaard	5,446	5.86
Hanson	5,239	5.64
Morrison	7,362	7.93
Forbes & Kriss	4,882	5.97

Standards for Milk Production per Pound of Milk

	Percentage of Fat in Milk				
	3%	4%	5%	6%	7%
Net energy "for fattening" in Calories					
Armsby	214	263	315	361	408
Mollgaard	235	281	326	368	406
Hanson	248	281	314	...	...
Morrison	250	305	355	401	445
Forbes & Kriss	233	279	325	363	403
Digestible nutrients in pounds					
Armsby	0.230	0.285	0.339	0.388	0.439
Mollgaard	0.252	0.302	0.351	0.396	0.437
Hanson	0.267	0.302	0.339	.....	.....
Morrison	0.271	0.328	0.382	0.432	0.480
Forbes & Kriss	0.251	0.300	0.350	0.393	0.433

The net-energy values "for fattening" given above from Kellner, Hanson, and Morrison were computed by Kriss from Kellner's "starch values," Hanson's "food units," and Morrison's "digestible nutrients," respectively; 1 kg. of starch value is taken to be equivalent to 2,356 Cal. of net energy "for fattening"; 1 food unit (originally representing 1 kg. of barley or 1 kg. of dry matter of roots) is considered as equivalent to 0.7 kg. starch value, or to 1,650 Cal. net energy "for fattening" or 2,100 Cal. for milk energy; 1 kg. of digestible "true" protein is equivalent to 2,233 Cal. of net energy for fattening (Kellner, Mollgaard). According to Forbes & Kriss (75) 1 pound of digestible nutrients is equivalent to 1,616 Cal. of metabolizable energy or 929

Cal. of net energy for fattening; or 1,000 Cal. of metabolizable energy is equivalent to 693 Cal. for milk energy or 575 Cal. of net energy for fattening. Forbes & Kriss (75) give the following important equivalents in term of Calories of metabolizable energy per pound of digestible nutrients: timothy, clover, or mixed hay, 1,527 Cal. per pound; alfalfa or soy-bean hay, 1,608; corn (maize) silage, 1,662; mixed rations (grain and hay), 1,616 Cal. per pound.

Fingerling and associates (76) found that 100 grams of maize silage, with a dry matter content of 23.03 per cent, is equivalent to 13.6 grams of starch for production purposes in cattle. Fingerling (77) also reported that in case of swine (growing or adult) 1 gram of digested potato starch (N-free) gave an energy storage in the animals of 3.411 Cal. as compared to Kellner's values for ruminants of 2.36 Cal. He concluded therefore that the pig is about 30 per cent more efficient in converting "pure nutrients" to fat than are ruminants, and that this efficiency is independent of age of the animal (in addition to the production ration, the animals were supplied with a basal ration to cover the maintenance needs). Fingerling (78) reported that the starch equivalent of maize silage grown in a wet year was lower than that grown in a dry year.

Fraps (79) published tables of "production energy" of 20 feeding stuffs for sheep. This production energy is said to correspond to Armsby's net energy. One pound gain in weight of fattening sheep requires 2,600 Cal. of productive energy. The productive energy of alfalfa was increased 14 per cent by grinding. The productive values of corn fodder and of oat straw were greater in balanced than in unbalanced rations.

Forbes & Voris (80) investigated the economy of conversion of food energy into milk energy by the dairy cow. They found that 9 Holstein cows of average weight 521 kg. (1,146 lbs.), producing about 5,356 kg. of milk per lactation period, transformed approximately 21 per cent of their food energy into milk energy. The most efficient cow converted 23.4 per cent and the least efficient cow 18 per cent of the food energy into milk energy.

Kahlenberg & Voris (81), in Forbes's laboratory, investigated the energy value of milk of Holstein-Friesian cows. They covered the entire period of lactation of 12 cows, including 134 samples, each sample being a 28-day composite. They found the regression equation of energy on fat to be $E = 128.55\ (1.886 + f)$; for energy on fat and non-fat solids $E = 183\ (3.04 - 0.31t + f)$. Andersen, Langmack &

Winther (82) found the caloric value of Danish cow's milk to be represented by the formula $C = 300 + 113.5F \pm 8$, where C represents Cal. per kg. of milk and F is fat content.

Overman & Gaines (83) determined the caloric value of cow's milk and milk constituents thus: 1 kg. of milk containing 4 per cent of fat and 37 grams of protein (50 mg. of protein per Cal.) equals 750 Cal. or perhaps slightly more. The caloric values per gram are 9.253 ± 0.065 for milk fat; 5.853 ± 0.127 for protein; 3.693 ± 0.059 for lactose. For practical purposes milk of any breed should be converted to milk of 4 per cent fat by the formula: $FCM = 0.4M + 15F$, in which FCM is milk containing 4 per cent of fat, M is the weight of milk, F is the weight of fat, all in the same units. The caloric values of milk from different breeds of cattle are given by the following equations: Jersey, $E = 106.98\ (3.15 + f)$; Holstein, $E = 128.19\ (1.99 + f)$; Guernsey, $E = 116.80\ (2.52 + f)$; Ayrshire, $E = 121.00\ (2.20 + f)$; Crossbred, $E = 116.11\ (2.61 + f)$, where E is Cal. per kg. of milk, f is percentage of fat in the milk. Assuming with Fredericksen that the ratio of protein content to the energy value of milk is nearly the same regardless of the natural fat content, the milligrams of milk protein per Cal. are given by the following values: Holstein, $46.49 + 0.46f$; Crossbred, $49.68 - 0.61f$; Guernsey, $48.89 - 0.83f$; Ayrshire, $56.29 - 2.32f$; Jersey, $55.59 - 2.32f$; or roughly, the protein-energy ratio is within the limits of 43 and 49 milligrams of protein per Cal. For a given fat percentage, Holstein milk has more protein per Cal. than the Crossbred; the Crossbred more than the Guernsey; the Guernsey more than the Jersey.

As regards energy allowance for milk of lactating dairy cattle, Overman & Gaines suggested that the milk yield be first converted to milk containing 4 per cent fat by Gaines's formula cited above, so that 1 kg. of the 4 per cent milk contains 750 Cal. and 37 grams of protein. They then suggest that a 100 per cent margin be allowed for the milk calories and 50 per cent margin for protein in computing the requirements for digestible energy and protein. The energy requirements for 1 kg. of milk will then be 1,500 Cal. of digestible food energy and 55 grams of digestible protein. According to this suggestion the only necessary data for energy feeding of dairy cattle for milk production would be (*a*) energy content of cattle feeds; (*b*) content of protein; and (*c*) coefficients of digestibility of these two items. This suggestion unfortunately does not eliminate the difficulties due to the disturbing factors previously cited (the influence of plane of nutrition, mutual

influence of the component foods in the ration, etc.); it merely overlooks them. However, it does make it appear unnecessary to have different feeding standards for milks containing different percentages of fat.

Humans.—Attempts to formulate food standards for humans are naturally more empirical, based on observations rather than experiments, and the results are in general more fragmentary. The caloric values of the foods are usually estimated on the basis of their "physiological fuel values" (equivalent to "metabolizable energy") assuming that such "fuel values" are 4 Cal. per gram for protein and carbohydrates, and 9 Cal. per gram for fats. The concept of net energy is not at all used in human nutrition.

Wait & Roberts (84) weighed the food intake of 52 well-nourished girls ten to sixteen years of age during a period of one week. The daily individual caloric consumption ranged from 1,649 to 2,925 (protein 7.72 gm., fat 94.2 gm., carbohydrates 300 gm.) or 53.3 Cal. per kilogram, or 15.5 Cal. per centimeter of height. The intake per kilogram decreased from 68 Cal. at ten years to 37 Cal. at sixteen years. Caloric intake per centimeter of height remained approximately constant. The daily variation of energy intake ranged from 10 to 181 per cent (average 49). In 27 per cent of the cases the day of maximum intake followed or preceded that of minimum intake.

Coons & Schieffelbusch (85) found that Oklahoma women have a low food intake, averaging only 67 per cent (796 Cal.) in excess of the basal values. The protein consumed averaged 1.1 grams per kilo of body weight (range 0.7–1.2) and furnished 11 per cent of the total calories. The authors think that the food should furnish 1,000 Cal. in excess of the basal metabolism.

Young (86) measured the caloric intake and output of 172 male medical students, aged twenty to twenty-five years, average weight 155.5 pounds. The average intake was 2,808, while the output was 2,592 Cal., the surplus being 85 per cent, while 14.6 per cent of the calories were derived from protein (100 gm.); 38.5 per cent from fat (11 gm.); 17.3 per cent from carbohydrates (328 gm.).

Cathcart & Murray (87) determined the caloric intake of 154 families in a non-industrial community in England. The average daily intake per man was 3,119 Cal., which is about 12 per cent above the customary standard. This represents not a flat increase for all nutrients, but an increase of 19 per cent for fat. In the case of women, on the other hand, the intake was 21 per cent below the man's standard

instead of the conventional 17 per cent below man's and over 29 per cent below the actual man value, so that the man consumed 36 per cent of the total calories of the family diet. The increased protein intake of the man was compensated by a decreased protein intake in the woman who obtained 24 per cent less of protein and fat and 19 per cent less of carbohydrates than the man. The percentage distribution of the constituents was protein 11, fat 35, carbohydrates 54. The high fat and low protein content was apparently due to choice. As the income increased, the caloric consumption of the family became greater, due mainly to an increased proportion of fat and to a less extent of protein in the diet. The calory intake was apparently greater for sedentary than manual workers. The man to woman ratio of intake was found to be 1:0.66; 1:0.70; 1:0.71; 1:0.69; and 1:0.76, instead of the customary 1:0.83. Tigersted's data on 25 families gave a ratio of 1:0.66.

Benedict & Farr (88) measured the gross energy value of mixed meals in sorority houses, cafeterias, and restaurants in a small college town in New Hampshire. In all cases the gross energy value per gram of air-dry food in which visible fat was absent was found to be 4.7 Cal. The edible waste from these sororities averaged 5.0 Cal. per gram of air-dry matter. The energy factor for samples containing visible fat was appreciably greater than 5. It is stated that the energy content of edible table waste (as distinguished from refuse) can be calculated with reasonable accuracy by multiplying the air-dry weight in grams by the factor 5 if visible fat is not present. If there is visible fat present and it can be removed, its energy value should be calculated separately by multiplying its weight in grams by the factor 9. The gross caloric values of individual articles are also given from dill pickles (0.13 Cal. per gm. as purchased) to peanuts (7.2 Cal. per gm.); and from whole wheat bread and white bread (4.0 Cal. per gm., air-dried) to muffins (4.6 Cal. per gm., air-dried).

Stewart, Boyd & De (89) published caloric values for cow and buffalo milk, rice, flours, lentils, etc., also the caloric value of hospital diets of Hindus, Mohammedans, and Europeans in India.

Brookens (90) found that the heat of combustion of glycogen is 3,787.7 cal. per 0.9 gm. of anhydride obtained from a specimen of *Mytilus* glycogen, purified by electrodialysis and of very low ash content.

Neuberg & Hofmann (91) found that the caloric value of pure methylglyoxal is 4,801.8 cal. per gram, and the heat of solution and

hydration 143.1 cal. per gram. The figures for phenylglyoxal were, respectively, 5,950.1 and —31.6; for phenylglyoxylic acid, 5,620.9 and —16.25; for methylglyoxal monoxime, 4,625.4 and —44.6; and for phenylglyoxal monoxime, 5,508.6 cal. per gram.

Gigon, Noverraz & Hoffmann (92) determined the caloric values of blood constituents and found these to vary (for rabbit, pig, cow, horse, man, in terms of cal. per gram of dry substance) from 5,200 to 5,500 for whole blood; 4,900 to 5,200 for serum; 4,800 to 5,200 for plasma; and from 5,400 to 5,600 for red blood corpuscles.

Cathcart & Cuthbertson (93) analyzed the fat from various regions of human bodies with the following results for caloric values: abdominal fat, 9.505 Cal. per gm.; liver fat, 9.11; muscle fat, 9.23. The R.Q.'s were: abdominal fat 0.711, liver fat 0.719, muscle fat 0.717. Values were also obtained for hydrogen, carbon, and oxygen content. The iodine numbers were determined. On the basis of these analyses, modifications are suggested for the caloric values given in the Zuntz-Schumberg-Lusk table for 1 liter of oxygen at different R.Q.'s.

Factors Influencing the Over-All Efficiency of Animals as Transformers of Energy

To avoid repetition we may define at the outset the over-all *energy efficiency* of a productive process (as growth, fattening, milk secretion, egg production) by the ratio of the energy gained in the given product during a given time to the energy consumed during the same time; the *efficiency index* as some ratio which when multiplied by a conversion factor will give the actual over-all efficiency; the *partial efficiency* or *net efficiency* as the ratio of the energy in the product to the energy in the diet needed for its production but not including the overhead cost of maintenance. We shall review some material on the influence of size of animal, temperature of environment, and plane of nutrition on the over-all productive efficiency indices.

Influence of size of animal on the efficiency index.—Other conditions being the same, should a large or small cow be a more efficient producer of milk? Should a large or small hen be a more efficient producer of eggs? Is a rabbit a more or less efficient producer of meat than a steer? To answer such questions one must know (*a*) the relative partial (or net) efficiencies of these processes, and (*b*) the relative costs of maintenance since the over-all efficiency (which is the prime practical problem) is a function of *a* and *b*. If it is assumed that the partial efficiencies (*a*) of a given process (as milk production) are the

same in large and small animals, then the over-all efficiencies (b) are functions of the maintenance costs. If the energy cost of maintenance increases with the 0.7 power of weight, as does roughly the basal metabolism, then the problem narrows down as to whether or not the energy value of the milk produced likewise increases with the 0.7 power of live weight. If it does, then the efficiency is independent of live weight; if the energy value of the milk increases with a higher power of weight than 0.7, then the over-all efficiency increases with increasing weight; if it increases with a power of weight lower than 0.7, then the efficiency decreases with increasing weight.

Kleiber (94) believes that the over-all efficiency is independent of live weight. His belief is based on rather theoretical considerations as follows: Kleiber found that the ratio of U, the maximum total food energy which an animal consumes per day, to B, its basal metabolism, is nearly the same in baby chicks, rabbits, sheep, and steers. In fact, according to Kleiber, individual differences in this ratio (called "relative feed capacity") among steers are greater than between steers and baby chicks. Large as well as small animals can consume food energy of four to six times their basal metabolism energy. Kleiber then reasons that the total efficiency of food utilization in two animals is the same if with equal partial efficiencies, k, their relative food capacity is the same. He defines k as the ratio of a net-energy increment to the corresponding increment of food energy (net efficiency). He generalized this statement by the equation $W = k - \left[\frac{B}{U}\right]$, in which W is the over-all efficiency and the other terms are defined as previously.

The literature does not yield much evidence which would enable one to judge the correctness of Kleiber's deductions. Thus, while McDowell (95) found (on the basis of 109,116 lactation records) that large cows are more efficient milk producers than small cows, Gaines (96) in a later paper found that small cows are more efficient. However, in both of these citations the data were defective in some respects. Jordan (97) in a very widely quoted statement found that the efficiency as computed from the percentage ratio of "edible solids" in the product to "digestible organic matter" consumed is 18 per cent for milk production in dairy cattle, 15.6 per cent for pork production, 8.1 per cent for veal production, 5.1 per cent for (fowls) egg production, 4.2 per cent for chicken production, 2.5 per cent for steer-beef, and 2.6 per cent for mutton production. However, in these instances the partial efficiencies for production may be different for the several

processes and in this sense do not contradict Kleiber's conclusions. Kleiber in his analysis indicated that the maximum ratio of fat production to basal metabolism is the same in rabbits as in steers, and that similar ratios hold for milk production in the large Holstein as in small Jersey cattle, as also in swine. The available data, including those cited by Kleiber, are still insufficient, and, as he points out, the conclusions are based on theoretical considerations to the effect that the productive abilities of animals tend to vary with the ¾ power of live weight in the same manner as basal metabolism tends to vary with the ¾ power of live weight. An extreme deviation from the Kleiber rule would be the exceptionally low over-all efficiency of growth in humans as compared to that of other species. Lusk in his book (13) discussed the well-known fact, discovered by Rubner, that the efficiency of growth in humans is less than in other species, and this difference was explained by Lusk by the fact that the rate of growth in humans is relatively less than in other species and therefore the energy cost of maintenance during a given gain in weight is greater.

Unpublished material in the reviewer's laboratory on the relation between egg production and body size in the domestic fowl contradicts Kleiber's conclusions. While the basal metabolism of the fowl was found to vary with the 0.68 power of the body weight ($Q = 70M^{0.68}$ where Q is the heat production per day and M is the body weight in kg.), the weight of annual egg production tends to vary exponentially (for the heavier breeds, $Y = 23.7 - 673e^{-1.16x}$, where Y is the annual egg production for body weight x), or indeed to decline somewhat for heavier weights above a certain weight limit. Assuming that the partial efficiencies of egg production are independent of body size, then the over-all efficiency index, which is represented by the ratio of egg production to body weight raised to the 0.68 power, declines with increasing weight; or it reaches a maximum for relatively light birds (3¾ pounds in the Leghorns, and 4¾ pounds in the larger breeds); then the over-all efficiency declines at the rate of 10 per cent to 18 per cent per pound increase in live weight. Incidentally, the weight of the individual eggs tends to increase with the 0.15 power of the weight of the birds (as compared to the estimated increase in the energy cost of maintenance with the 0.68 power of weight of the birds). The annual number of eggs tends to decrease with increasing live weight above a certain relatively low live weight. Data on milk production in the reviewer's laboratory indicate that the milk energy tends to increase with the 0.3 to 0.4 power of the live weight of cattle so that the efficiency

index of course declines with increasing weight. Certain genetic considerations complicate this problem to an extent which makes it impossible to show in this place that Kleiber's conclusions cannot be accepted without reserve.

Influence of environmental temperature on the efficiency index.—Kleiber & Dougherty (98) measured the metabolism and growth efficiency in chicks 6 to 15 days of age in "climatic cabinets" having temperatures of 70°, 80°, 90°, 100°, and 105° F. The amount of food eaten increased linearly with declining temperatures. The daily rate of growth increased with decreasing environmental temperature from 2.74 gm. at 105° to 4.88 gm. at 70° F. (this growth rate was 4.2 to 6.5 per cent of the respective body weights). But the over-all efficiency was a maximum at 90°, when the efficiency was 34 per cent of the "available" food energy; it dropped to 16 per cent at 70°, and to 29 per cent at 105°. The drop in efficiency from 90° to 70° is understandable since the CO_2 production (maintenance) increased with decreasing temperature from 2.95 liters of CO_2 per chick per day at 105° to 6.25 liters at 70°; or from 23 liters per kg.$^{3/4}$ at 105° to 43.4 per kg.$^{3/4}$ at 70° (R.Q. 1.04 during day and 0.92 during night). The basal metabolism decreased from 60 Cal. per kg.$^{3/4}$ at 105° to 128 Cal. per kg.$^{3/4}$ at 70°. No indication of a "critical temperature" was found. The partial efficiency (increase in body energy to corresponding increase in food energy) reached the maximum of 72 per cent at 100° and decreased to 57 per cent at 70°.

Giaja & Gelineo (99) investigated the influence of environmental temperature on the growth of rats. Litter mates were raised at different temperatures ranging from 7° to 30° C. The animals grew much more rapidly at the lower than higher temperatures. Thus at age 90 days the females and males were respectively 27 and 25 per cent heavier at the lower temperature. The effect of temperature is thus the opposite for homoiotherms than for poikilotherms. But since the metabolism is accelerated in homoiotherms and retarded in poikilotherms by lowering the temperature, the conclusion is therefore drawn that the rate of growth tends to be proportional to the metabolic intensity.

Influence of the plane of nutrition on efficiency of growth.—As pointed out by Wiegner & Ghoneim (68) on the basis of theoretical considerations, the successive increases in the net energy of successive increments of food ingested must decline, and this decline is probably exponential. This, of course, implies that the successive increases in

body energy per unit of corresponding food energy (which is the definition of net or partial efficiency) must likewise decline. This is merely another statement of the "law of diminishing increments" so much discussed by economists and also by plant physiologists which Mitscherlich (100) represented by the equation $Y = H\ (1 - e^{-kx})$ in which Y is the growth of the plant with increasing quantity, x, of plant nutrients. Jull, Titus & Hendricks (101) have investigated the quantitative relation between growth and food consumption of the domestic fowl, and found this law to hold true. These authors write the differential equation in the form $\frac{dW}{dF} = K\ (A - W)$, and the integrated equation in the form of $W = A - Be^{-kF}$, in which W is the live weight of the animal consuming the quantity F of food. In other words, the net efficiency of growth declines exponentially with increasing food consumption. Wiegner's equation may indeed be written in the form used by Hendricks. One might say that the efficiency of growth increases with decreasing food consumption because the net energy decreases with increasing food consumption. The investigations of Wiegner and the Jull groups of workers were carried out somewhat simultaneously but entirely independently and using quite different methods of investigation.

Heat Production and Influencing Factors

We shall first discuss the so-called basal metabolism, then certain superimposed factors influencing heat production. The literature on basal metabolism, including that on units of reference, up to 1930 was reviewed by Du Bois (102).

Units of reference.—Workers in the field of energy metabolism are not agreed on a unit of reference. Basal metabolism is said to vary with surface area which is roughly true for normal "mature" homoiotherms within a species. Terroine & Delpech (103) emphasized that it is true only for animals within a species and only in a thermally neutral environment. According to Kleiber (104) the so-called surface law cannot be explained by Fourier's law of heat flow, as the specific insulation can be varied by the animal, although, of course, there are limits to such variations; nor can it be explained by the nutritive surfaces, cell surfaces, or protoplasmic structures; or by the composition of the body, inert fat, "active protoplasm" and amount of blood, although, of course, they all affect it. This relationship is governed by the organism as a whole and cannot be derived from a summation of

the vital functions of the cells or other parts of the body. In brief, there is no theoretical evidence to indicate that the metabolism of animals should be related to their surface areas. But the most serious objection against the "surface law" is that we do not know how to measure surface area so that the results of different investigators shall be reproducible within reasonable limits of errors. As an illustration of this difficulty the reviewer (105) has compared the results obtained on measuring the surface area of the rat by Mitchell & Carman (106), by Lee (106), and by Diak (106), and found that the average difference between the areas as determined by Mitchell and Lee is 25 per cent, between Lee and Diak, 30 per cent; between Mitchell and Diak 60 per cent. If no better agreement can be obtained in measuring the skin areas of dead rats after the removal of the hair, dipping the bodies into varnish, carefully removing the hardened skin and measuring its area after flattening it, then what chances are there of obtaining reasonable agreement on living animals of different species?

Since area increases with a fractional power of weight, and since it is very difficult to measure the surface area, then it would seem simpler to relate metabolism directly to a fractional power of weight of the animal rather than to area. According to Meeh, the surface area increases with the ⅔ power of weight. The present reviewer (105, 107) and Kleiber found the BMR to vary with a rather higher power (between 0.68 and 0.75) of weight. These authors suggested therefore that at least for special comparisons metabolism be expressed per unit of weight raised to an agreed power. Kleiber (94) adopted 0.75 as the power, while the present reviewer is inclined to a lower value, perhaps 0.70. The coefficient in such a power equation is of the order of 70 to 73.

Benedict (108) appears to accept the ⅔ exponent for evaluating surface area and suggests that the value of 10 be agreed upon for the coefficient; so that Benedict's formula for relating surface to weight is $S = 10\,W^{⅔}$. The present reviewer (105, 107) has however shown that surface is not usually directly proportional to weight raised to the ⅔ power, and, moreover, that the increase in heat production does not vary with the same power of weight as does surface area.

The foregoing proposal is not meant for the study of human metabolism, except for comparing the metabolism of humans with other species, because in the case of human metabolism it is usually assumed that the Du Bois "linear" formula should be used for computing the surface area, while there is no such agreement for species comparisons.

But even in the case of humans some objections have been advanced against the Du Bois formula. Thus Jenkins (109) maintains that the Dreyer standards, based on observed weight, are the soundest followed by the Mayo standards. The Harris-Benedict prediction tables are excellent for adults, but not for children. The pioneer standards of Du Bois are not as sound as the aforementioned.

Kleiber (104) proposed the following equations to represent the basal metabolism in humans: for women, $M = 67.4\ W^{3/4}\ [1 + 0.004\ (30 - a) + 0.018\ (s = 43.4)]$; for men, $M = 71.2\ W^{3/4}\ [1 + 0.004\ (30 - a) + 0.010\ (s - 43.4)]$; M represents the basal metabolism in Cal. per day; W, body weight in kilograms; a, age in years; s, specific stature $= \frac{\text{stature in cm.}}{\text{weight}^{1/3}}$. According to Kleiber the advantage of this equation as compared with the empirical Harris-Benedict equation is that each term in the former has a physiological meaning.

Bohnenkamp and associates (110) claim that they have reduced the study of basal metabolism to terms of Lambert's equation, the Stefan-Boltzmann relation, and the universal radiation constant. They consider the geometrical surface applies to loss of energy by evaporation, conduction, and convection, and a special surface, equal to $4\ \pi$ times the square of the electrical capacity of the body, applies to radiated heat. These investigators measured photographically the projected surfaces of several individuals, and the intensity of radiation from different skin areas by a special thermopile. They found women and obese subjects to show lower intensities of radiation than men and individuals of normal weight. The Stefan-Boltzmann law relates the energy radiated to the area of the effective radiating surface, the temperature of the radiating body and the temperature of the surroundings. Methods are described for computing by this law the energy lost by radiation, and the influence of clothing thereon. Bohnenkamp and co-workers believe that body weight is not a good unit of reference since different tissues have different metabolic intensities. They use instead specific gravity which varies mainly with the amount of fat in the body which is intimately related with the "average skin temperature," and from which the "average skin temperature" is indeed deduced. From this average skin temperature, and the effective radiating surface (which is about 85 per cent of the surface in the Du Bois height-weight chart), the energy loss by radiation is calculated according to the Stefan-Boltzmann law. This is supple-

mented by the energy lost through evaporation (computed from the Benedict-Root equation) and the energy lost by conduction and convection. Truer values, according to the authors, are thus obtained for individuals of abnormal figure. Heller (111), however, maintains that Bohnenkamp's energy law is not tenable, referring to evidence which he previously published. Pfleiderer (112) also criticized Bohnenkamp's work, to which the latter replied in the same issue.

Deighton (113) believes in the essential rightness of the surface law, but he finds the present methods of estimating surface unsatisfactory so he proposes a new method of measuring surface area in a definite standing position involving a photographic technique. This author (114) has also reviewed extensively the literature on the physical factors in body-temperature maintenance and heat elimination and expressed himself favorably to Bohnenkamp's theory.

Mitchell (115) agrees that surface area is not a definite measurement and that the heat produced per square meter of body surface possesses no absolute meaning, but the method is highly useful and significant for comparative purposes.

Topper & Mulier (116) investigated the effect on basal metabolism of ligating the lower extremities in humans and concluded that the basal metabolism depends on the amount of active protoplasmic tissue rather than on the body surface.

The following new data appeared on surface areas of animals: Mitchell (117) found that the surface area of White Leghorn chickens is represented by the equation $S = 8.19\,W^{0.705}$ (area in sq. cm., weight in grams). Southgate (118) proposed a new method for determination of the surface area of birds. Lee & Fox (119) measured the surface area in the monkey, *Macacus rhesus,* and found the relation to be $S = 11.7\,W^{\frac{2}{3}}$. These authors (120) also measured the skin areas of mice, guinea pigs, and woodchucks. Ritzman & Colovos (121) measured the surface area of sheep. For lambs they found the relation to be $S = 139\,W^{0.501}$; for yearlings, $S = 147\,W^{0.520}$; for adults, $S = 126\,W^{0.556}$; for the entire flock of 115 animals, $S = 124\,W^{0.561}$. Lines (122) measured the area of Australian Merino sheep, the skin of which is thrown up in large folds around the neck. The area was measured by means of a planimeter after slaughtering the animals. Using the ⅔ power, the formula relating area, A, to live weight, W (food removed for 48 hours previously), is $A = 0.0909\,W^{\frac{2}{3}}$; but the formula $A = 0.121\,W^{0.59}$ gave better approximation for the animals of all ages. Smuts (123) measured the surface areas of the mouse,

guinea pig, and rabbit, and found the coefficient in Meeh's formula to be 7.76, 7.35, and 9.18 respectively. Mardones (124) measured the surface areas of rats, and found the formula to be, $A = 78 + 2.1\,W - 0.0029\,W^2$. Benedict & Fox (125) computed the areas of small birds from the formula, $S = 10\,W^{2/3}$; of mice (126) from the formula, $S = 9\,W^{2/3}$; of sheared sheep (108), $S = 8.4\,W^{2/3}$; of snakes (108), $S = 12.5\,W^{2/3}$. Boyd, Scammon & Donovan (127) measured the surface areas of small children with the aid of plaster casts, finding the formula, $S = 1008\,W^{0.692}$, to represent the results almost as well as $S = W^{0.575}\,H^{0.275}$. Necheles (128) tested the usefulness of the Du Bois height-weight formula on Chinese (65 men and 63 women) with good agreement between the height-weight formula and linear measurements of Du Bois and Stevenson.

Basal metabolism of humans.—Janet & Bochet (129) presented metabolism data on infants from birth to seven months obtained by their *sac-respiratoire* method. The metabolism per square meter increased with age. The increased heat production due to feeding was compensated by the lowered metabolism due to the infants falling asleep after feeding. Schadow (130) found that the BMR of premature infants is less than in normal infants. In the first month it was —9 per cent on the weight basis and —26 per cent on the area basis; in the second month it was —4 and —23 per cent. The rise in metabolism caused by ingesting milk averaged 5 per cent for the premature infants and 8.5 per cent for the normal infants. The total metabolism (including that due to muscular activity and SDA) is lower in the premature infants as follows: first week —33 per cent on weight basis and —58 per cent on area basis; first month —29 per cent and —46 per cent; second month —19 per cent and —38.8 per cent; third month —0 and —20 per cent. It is suggested that during the first month the daily caloric intake should be between 100 and 120 Cal. per kilogram. Schlossmann & Murschhauser (131) found that in normal infants O_2 consumption averaged 27.4 cc. per minute and R.Q. 0.828; in premature, O_2 consumption was 6.2–12.7 cc. and R.Q. 0.852– 0.891. Results confirm Schadow's work. The low values for premature infants are attributed to their low body temperature.

Topper & Mulier (132) found an increase in the basal metabolic rate in the prepubescent period, reaching its maximum around the time when catamenia was established in girls (12–13 years), and sexual maturity in boys (13–14 years), with a subsequent decline afterward,

especially in girls. There was no uniformity in the height of the increased metabolism (about 14 per cent), or in the duration of the increase. This increase in metabolism often coincides with symptoms of enlarged thyroids, tremor, nervousness, vasomotor instability and cardiac murmur, all of which disappear as the basal metabolic rate is returned to normal and puberty is well established. As this increase occurs at a given physiological rather than chronological age, it is therefore difficult to establish normal standards for this period. Topper (133) investigated the metabolism of 20 children with retarded growth. All tests showed that the basal rate exceeded the normal when referred to standards for height, sitting height, weight, and surface area, but agreed with standards according to age.

Albergo (134) studied the BMR and SDA of 22 persons ranging from 59 to 82 years. He found a decline in BMR and in SDA with increasing age. Benedict & Meyer (135) measured the BMR of 23 women, 66–86 years of age, weights 32–72 kg. The BMR decreased with age. The average for women of 78 years and over was close to 1,000 Cal. per 24 hours. There was no association between blood pressure and BMR. The women having the greatest vigor had the lowest BMR partly because they were lightest in weight. Brody & Procter (136) have treated these and other data mathematically and found that there is a slight but unmistakable decline in metabolism per unit weight with increasing age.

Biometric equations for basal metabolism in humans.—Wetzel (137) presented mathematical analyses and equations for the energetics of growth and starvation. Growth is viewed as a process of biological energy exchange, and the mathematical expression relates the "internal work of growth" (new cells), the "external work" (energy utilized and dissipated in synthetic processes), and "maintenance work." The equation is applied to humans for increase in weight with age (100-day conceptional age to 32 years), to changes of basal metabolism with age, to fasting data (by Benedict on Levanzin) showing that with a certain rate of loss of weight the heat liberation will be minimal and constant, and that the optimal rate of weight loss during starvation for minimal metabolism and hence for the best conservation of the body's reserve is 0.0064 kg. per day, and finally, to age curves of growth and heat production of bacterial populations. The apparently complex mathematics in this paper are correct provided that the fundamental assumptions are right (the basic equation relating "energy of the source" to "internal work of growth," plus "exter-

nal work of growth," plus "work of maintenance" and especially on the constancy of E_e, the "energy of cellular syntheses per unit charge"). The equation in its final form is not fundamentally different from some growth equations previously proposed, being exponential; but on the surface it appears different, being written in hyperbolic form which is unusual in biological papers. The equation fits the data more satisfactorily than some of the other equations for the reason that it contains more terms than some of the equations previously proposed for this purpose. The importance of this contribution is necessarily dependent on the degree of correctness of the aforecited basic assumptions.

Jenkins (109) believes that each laboratory should determine its own BMR's on 25 normals for its normal population. Deviations of 10 to 17 per cent from the normals should be considered as doubtful. The author suggests that the BMR may be predicted from the basal pulse complex by the following regression equations: men, 0.534 pulse rate + 0.436 pulse pressure — 59.94; women, 0.497 pulse rate + 0.368 pulse pressure — 57.42. A rise of 1° F. in body temperature corresponds to a rise in BMR of 7 per cent in men and 5 per cent in women; a rise of 1° C. corresponds to increases of 12 per cent in men and 9 per cent in women. The author suggests that the BMR should be determined by more than one standard. Stark (138) gives the following prediction formula for girls of age 17 to 21 years: Cal./24 hours = 10.63 × wt. (kg.) + 3.23 × height (in cm.) + 184.61. The measured values were 8–15 per cent below the Du Bois and 6–10 per cent below the extrapolated Harris-Benedict predictions. Levine & Marples (139) from a biometric analysis of metabolism in infants confirmed the Benedict-Talbot standards; it is indicated that age per se is an apparently trivial influencing factor; sex during this period exerts no influence. A nomogram is presented for predicting the basal metabolism of normal infants from body weight and stature. Umber (140) found that the Read formula, $BMR = 0.75\ (PR + 0.74\ PP) - 72$, where PR is pulse rate, PP is pulse pressure, gave excellent agreement with basal metabolism determinations in 100 patients (52 of them suffering from Graves disease). There was an 8 per cent variation between observed and computed values when the metabolism increased due to SDA. Bruen (141) analyzed Bierrings' (142) and other data and published the results in the form of exponential prediction equations and prediction tables. This author also found a definite acceleration in BMR during puberty. Brody & Procter (107, 136)

found that during the post-natal period of active growth the heat production per unit weight, Q, is related to body weight, m, by the equation $Q/m = Ae^{-km} + C$; and that with respect to age, t, it is related rather less satisfactorily by the equation $\frac{Q}{m} = Ae^{-kt} + C$, in which e is the base of natural logarithms, A and k are the parameters of the equation. These equations represent the weight and age changes in "basal" or "resting" metabolism not only for humans but also for cattle, horses, sheep, swine, and rats. It may be noted in this connection that during fasting the live weight, m, declines exponentially with time, t, according to the equation $m = Ae^{-kt}$ (143).

Hendricks, Jull & Titus (144) deduced from the "diminishing increment" equation, $W = A - Be^{-kF}$, that the *total cost* of maintenance (including voluntary muscular activity) at any time is proportional to the product of the live weight of the animal and the amount of food ingested, or that the amount of food used for maintenance during the time the animal is consuming the increment of food, $F_2 - F_1$, is $(F_2 - F_1) - \frac{W_2 - W}{kA}$.

Basal metabolism data on farm animals.—Brody and associates (107, 136) measured the "basal" and "resting" metabolism of dairy cattle, beef cattle, horses, sheep, and swine from birth until about five years of age and of the domestic fowl from hatching to about two years of age. "Resting" metabolism differs from "basal" metabolism in including the heat increment of feeding. The results were summarized in the form of ratios of heat production to live weight $\left(\frac{Q}{m}\right)$ as functions of live weight, m, and of age, t, as explained in the preceding section. Normally-feeding cattle and swine had a heat production about 25 per cent above the fasting metabolism, sheep about 20 per cent above, and horses about 30 per cent above the basal levels. There is a very striking seasonal variation (with a maximum in May and minimum in November) in the metabolism of sheep which is independent of sex. Hall & Brody (145) measured the metabolism of well-trained cattle and sheep in the lying and standing positions and found the heat increment of quiet standing over lying is 9 per cent for medium-sized dairy cattle and sheep, and 13 per cent for an unusually heavy steer (weighing over a ton).

Ritzman & Benedict (146) found that measurements from the second to the fourth day after withdrawal of food, with a correction

for standing, represent satisfactory measures of basal metabolism in sheep (1163 Cal. per sq. m. per day). The energy of standing is 15 per cent over that of lying, while the stimulating effect of food results in an increase of 26 per cent. This later increase on a protein-poor carbohydrate-rich diet suggested to the authors that the path of absorption of carbohydrates in the ruminant is through the fermentative processes as postulated by Grouven. The BMR of the sexes was almost equal; castration in the male reduced it. The metabolism is relatively high at birth and early suckling periods, and declines with increasing age. A more elaborate report, including details of methods on these data, was published by Benedict & Ritzman (147). The average heat production for quiet, lying sheep, 6 months of age or older, is 1,275 Cal. per sq. m. per 24 hours. The R.Q. is 0.85 for young lambs, 7 hours after feeding, while for yearling or mature animals, this value is not reached until 24 hours after feeding. Usually an R.Q. of 0.73 is reached in yearlings or older 48 hours after feeding. The rectal temperature in sheep is about 1° above that in cattle. This is higher in lambs than adults. The range of rectal temperature is 38.8° to 40.4°. Heavy-wooled adults have a higher rectal temperature than light-wooled. There is a marked seasonal variation in the metabolism of sheep.

Lines & Peirce (148) measured the "standard metabolism" of Australian Merino sheep. "Standard" metabolism in sheep is defined as the heat production 48 hours after feeding while quietly resting in a standing position in an environment free from thermal influences. The metabolism thus defined was found to be considerably affected by the nutritive level and by the composition of the dietary taken previously to the determination. Recessions of 13 per cent were observed after changing from maintenance to slightly submaintenance for the course of one month. This is profoundly influenced by seasonal variations in fodder conditions of grazing Merinos. Standard metabolism of ewes on poor autumn feed was on an average 1,060 Cal. per 24 hours, and of wethers, 890 Cal. per sq. m. per 24 hours; but after excellent spring pasture the metabolism was 1,250 Cal. per sq. m. per 24 hours for both sexes. Standing was found to increase the standard metabolism between 5 and 10 per cent above that of the animals lying naturally. The R.Q. under these conditions was between 0.72 and 0.73; a few animals, however, had quotients between 0.80 and 0.85. No acetone was found in the urine of sheep having low R.Q.'s, but in

some cases of more prolonged fasting, up to 0.5 gm. per day of acetone bodies was excreted.

Mitchell and associates in the previously reported paper (69) found that the fasting heat production of their steer corrected to a "standard day" of 12 hours standing and 12 hours lying was from 1,554 to 1,855 Cal. per sq. m., depending on the method of computing surface area.

Forbes and associates obtained fasting metabolism data on cattle incidental to their net-energy studies. These metabolism data (as also Mitchell's) are not basal in the sense understood in human metabolism because the animals as confined in a respiration chamber for hours or days spend part of the time standing as well as lying and moving about. According to Forbes and associates (149) the heat production in cattle diminishes continuously as the fast progresses, and so no definite constant level can be reached. It is therefore suggested that for routine purposes the heat production of the first 24 hours after attainment of the non-protein R.Q. of fat be taken as the basal level. This level is attained by the second and certainly by the fourth day after the withdrawal of food.

Iftimesco, Nichita, Popesco & Tuschak (150) measured the energy metabolism of six races of fowls and concluded that the metabolism is proportional to surface area.

Basal metabolism data on laboratory animals.—Benedict & Fox (126) found that normal fasting laboratory-bred mice had a heat production averaging 640 Cal. per sq. m. of body surface per 24 hours at 28°.5 C. and 935 Cal. at 15°.7 C. (17 hours after feeding at minimum activity). The metabolism increased 3.6 per cent per degree of decrease in temperature, but it was higher at 32° than at 28°. Mice kept at 28° for long periods had metabolic values of 500 or even 400 Cal. per sq. m. These wide variations in the zone of thermal neutrality and metabolism are attributed to the incessant activities of this animal, and the tendency to subnormal body temperatures. It appears that what is called the basal metabolism in mice is of the order of 500 Cal. per sq. m. (the surface area in sq. m. $= 10\ W^{\frac{2}{3}}$). In hairless mice, thermal neutrality is at 34° when the basal metabolism averages 750 Cal. per sq. m.; at 28° the metabolism is of the order of 1,000 Cal. and at 16° it is three times that at 34° C. Davis & Van Dyke (151) found, at the University of Chicago, that male albino mice at 28° C. and 17 to 24 hours after feeding used on the average 38.5 liters of oxygen per kilogram per 24 hours when they were quiet and relaxed. The same

mice, studied in the Carnegie Nutrition Laboratory by a different technique under the same conditions, had an oxygen consumption of 40 liters. When the mice were sleeping, the Chicago investigation reported an oxygen consumption of 28.3 liters, and values as low as this were found at the Carnegie Nutrition Laboratory on a different group of mice. The great reduction in body temperature (1.6° C. to 3.5° C.) after the fast may in part explain the comparatively low metabolism. Benedict attributed the low values (minimum 390, average 557 Cal. per sq. m. per 24 hours) found by Davis & Van Dyke to the large respiration chamber used.

Chevillard (152) found that small adult mice liberate more heat per unit of surface than the larger; no simple relation exists between heat liberation and surface area. The smaller the mouse the greater is the effect of low environmental temperature in increasing the energy exchange. The ratio of water evaporation to oxygen consumption increases with increasing environmental temperature. Below thermal neutrality this ratio in mice is smaller than in larger laboratory animals, indicating that the smaller the animal the greater the importance of chemical, in comparison to physical, heat regulation.

Benedict, Horst & Mendel (153) measured the metabolism of two extraordinarily large albino male rats weighing 822 and 730 grams. As compared to average-sized rats their metabolism was lower per unit weight or area. Thus the metabolism per square meter per day was 708 Cal. for 307–396-gram rats, 664 for 407–562-gram rats, and 612 for the two unusually large rats. The two large rats were fasted for 57 and 38 days (when they were still living), losing respectively 46 and 55 per cent of their initial weight. Autopsy of the larger rat showed that after the 57-day fast he still had a liberal supply of fat. The total oxygen consumption decreased until about the 20th day of fast, but thereafter remained relatively constant. In the largest rat, minimum metabolic values of 377 and 381 Cal. per square meter per day were recorded on the 29th and 43d days of fast, which is said to be the lowest heat production that has been noted with warm-blooded animals having a rectal temperature of 37° C. Phillips, Ashworth & Brody (143) fasted a domestic fowl for 48 days and found a low value of 400 Cal. per square meter, or 35 Cal. per kilogram per day on the 20th day of fast.

Benedict & Petrik (154) found the BMR of wild rats at 24° C. to be 942 Cal. per sq. m. per 24 hours compared with 720 Cal. for

female and 800 Cal. for male albino rats. Wesson (155) found a low basal metabolism rate in rats of 719 Cal. per square meter and a fasting R.Q. of 0.722. These rats were previously kept for some days at 28° C. and in total darkness. He recommends this method for obtaining standard conditions. In this connection Wesson gives curves showing the BMR and R.Q. of normal rats for 45 hours after the ingestion of a definite carbohydrate test meal.

Benedict & Fox (125) measured the metabolism of canaries, parrakeets, wild English sparrows, and one song sparrow. They found it necessary to measure the birds after 6:00 or 7:00 P.M. in order to secure basal rest. Within the zone of thermal neutrality, male canaries had a somewhat higher metabolism than females; at 16° or below, the sex differences were obscured by the temperature effect. Ten to twelve hours after feeding were sufficient to reach a fat R.Q. under these basal conditions and at thermal neutrality. The basal metabolism of canaries and parrakeets averaged 718 Cal. per square meter and increased to 1,077 Cal. per sq. m. at 16°. Under similar conditions wild English sparrows had a heat production respectively of 656 and 914 Cal. per square meter.

Contrary to previous conceptions, these small birds with high cell temperatures (42° C.) have an unusually low metabolic rate per square meter.

Riddle, Nussmann & Benedict (156) measured the basal metabolism of tippler pigeons at 30° C. at various ages. From 40 days on, the metabolism per unit weight declined, first very rapidly, then at a lower rate, approaching about 1,000 Cal. per kilogram per day as the limiting value. Their data confirm in general the work of Ginglinger & Kayser (157). Riddle, Smith & Benedict (158) found that individuals of a feral, cold-avoiding, migratory species of dove, when reared in captivity, have a higher basal metabolism at 15°, 20°, and 30°, than that found in related, non-migratory, domestic doves and pigeons. At 20° C., the average BMR of migratory doves was 904, of ring doves 792, and of tippler pigeons 680 Cal. per sq. m. per 24 hours. The BMR of mourning doves is highest in September—males, 5–6 per cent higher than females. These data on pigeons have been discussed from a general point of view by the present reviewer (136). An interesting fact about pigeons and doves is that, unlike mammals, the metabolism per unit weight of the female is higher than of males, and the metabolism of the females declines less rapidly than of the males with increasing age.

Racial influences on BMR in humans.—This is a difficult problem to solve because the racial factor cannot be, or has not been, separated from other complicating factors, such, for example, as environmental temperature, humidity, muscular activity, diet and consequent amount and composition of body fat, manner of dress, amount of sunlight, and height of sun. Besides, the deviations from the so-called standards are at best small enough to be within the limits of error of the various techniques used for measuring BMR. Conclusions on the problem of racial differences in BMR must therefore be taken with considerable reserve.

Benedict (159) summarized his contributions on racial differences in BMR in terms of deviations from the Harris-Benedict standards as follows: Mayas in Yucatan, +5.2 to +8.4 per cent; South-Indian women in Madras, —17.4 per cent; Australian aboriginals, —14 per cent (males), and —16 per cent (females); Chinese girls living in Boston, —9 per cent. According to Benedict, climatic and dietary conditions do not explain these differences. A striking peculiarity of the Mayas is that their high BMR is associated with a low pulse rate (45 or lower), so that it is obvious that the high metabolism is not caused by a high heart-rate. Benedict & Meyer (160) found the metabolism of 18 normal American-born Chinese girls living in the United States, 12 to 22 years of age, to be on the average 9 per cent below the standards of American Girl Scouts of the same age, and 6 per cent below the Aub and Du Bois standards for American girls in this age range. The pulse-rates were somewhat lower than those observed with American girls. Necheles (161) recorded observations on Chinese (20 males and 20 females, 20 to 30 years) living in northern and southern China, and concluded that the metabolism of Chinese, at younger as well as adult ages, is lower than of Caucasians. The BMR of the younger Chinese tends to be relatively higher than of the older ones. On the basis of metabolism measurements during waking and natural sleep, Necheles concluded that the lower BMR of Orientals seems to be caused by their lower muscular tonus during waking. While the Chinese seem to be nearly as relaxed when awake as when asleep, this is rarely true for the Occidental. But Wang & Hawks (162) disagree with Benedict and Necheles. They measured the basal metabolism of Chinese children, of 5 to 14 years, reared or born in the United States; comparing their values with those of a group of American children of the same age range studied in the same laboratory under similar experimental con-

ditions, no marked difference was demonstrated. In a later paper Wang presents evidence that the difference between the basal metabolism of Chinese girls and that of American girls is less than that between the basal metabolism of American girls studied in different laboratories; and that the apparently low values found by Benedict & Meyer for American-born Chinese girls is due to the fact that, instead of comparing their results with American controls measured under the same conditions, they used the Girl Scouts and the Aub-Du Bois standards, which in Wang's opinion are too high for the majority of girls.

It is interesting to note that Kawata (163) found that normal Japanese gave BMR values agreeing with the Du Bois standards.

Pi-Suner (164) has pointed out that the basal metabolic rate of the Araucanian Mapuche, a race of Indians in Chile, is 9.8 per cent higher than the Harris-Benedict standard for men and 14.8 per cent for women in spite of very poor living conditions. Yet they exhibit a subnormal pulse-rate (60 in men and 67 in women). According to this author the racial factor is a more important influencing factor than the customary nutritional or climatic factors.

Mukherje & Gupta (165) found that the BMR of Bengali men is 13.3 per cent below the Aub–Du Bois standard. Banerji (166) found that the BMR of 145 prisoners of the district jail, Lucknow, in the United Provinces of Agra and Oudh, is 6.9 per cent below European and American standards. The author attributes the low BMR to environmental temperature and barometric pressure, but mainly to humidity. Krishan (167) found the basal metabolism of students in Madras to be below those of Western students by 8 to 12 per cent for men and 12 to 18 per cent for women (depending on the standards used). This is explained by greater muscular relaxation in hot climates and the low protein content of food eaten. Note is made of a rise in metabolism just before the onset and in the second week after menstruation.

Ocampo, Cordero & Conception (168) found that the BMR of 88 Philippine men (aged 20–30 years) was —7.8 per cent and of 16 women —9.3 per cent of the Aub–Du Bois standard. Similar results were obtained on Philippine residents in the United States, indicating that climate is not an influencing factor.

Turner (169) reported the BMR and vital capacity data for 28 Syrian women and 7 "control" American, English, or German women, who were residents of Syria. The BMR of the Syrian

women was —3 per cent and of the "controls" —6 per cent of the Aub–Du Bois standards.

Wardlaw & Lawrence (170) found that, while the BMR of Australian male aborigines in a hot, moist climate is 69.3 per cent of the Aub–Du Bois standard, that in a cool, dry climate is 87.6 per cent of the same standard. When physically inactive, the BMR of aborigines can sink to values much lower than those observed among white subjects. Hicks & Matters (171) succeeded in measuring the oxygen consumption of 45 natives in Central Australia at temperatures ranging from —2° to +10° C. in the early morning. The only source of heat was the smoldering remains of fires, the natives being naked and exposed in the open. Metabolic rates as high as +90 per cent were recorded, and were correlated with skin and body temperatures, and low pulse- and respiration-rates. The latter are explained as being due to increased output per beat and increased tidal air. Body-surface measurements by the gummed paper-strip method of Takeya gave results agreeing within 5 per cent with those obtained by application of the height-weight formula. Blood-pressure even in old subjects did not exceed 138 mm. Hg and averaged 110 mm. The waste of energy as heat is significant in view of the difficulty of obtaining food in this arid country, and has been shown by more recent work of Hicks *et al.* (in publication) to be offset by low metabolic rates during the heat of the day when winter shade temperatures reach 98° C. The oxygen consumption falls rapidly as the sun rises. The SDA of protein is normal and the R.Q. approaches unity after a meal. The apparently wasteful use of food resulting from absence of clothing or habitation during the winter nights is accounted for by the necessarily nomadic life of the native, who must travel great distances in search of game and water, and to whom implements and clothing would be a physiological hindrance during the hot days. Natives accustomed to European clothing show oxygen uptakes similar to those of Europeans.

Radsma & Streef (172) found that residence in the tropics reduces the BMR of Europeans 6.3 per cent below the Harris-Benedict standard and 5.7 per cent below the Du Bois standard. This is not due to a reduced protein intake, although increasing the protein intake increases the BMR.

Heinbecker (173) found that the BMR of Eskimos (Baffin Island) living on a mixed diet was similar to that of persons living on a like diet in temperate zones. The R.Q. during fasting was very

low (0.7). It is suggested that the Eskimo can oxidize fats more completely than can subjects in temperate zones.

It is interesting to note in this connection that it was observed (174) that Eskimo women are subject to an annual menopause and that men are impotent in winter. Such sterility is obviously not a racial peculiarity since the European members of the North Greenland expedition (174) were likewise affected. (May not the excessive clothing continuously worn in the arctic region raise the environmental temperature of the scrotum to a level causing impotence? It is well established that temperature elevation about the scrotum causes sterility.) It is known (175) that ovulation in birds is very sensitive to light and temperature. On the other hand, Maignon & Guilhoun (176) report a seasonal variation (maximum in May) in the BMR of dogs kept at a constant temperature. Riml & Wolf (177) found that seasonal variations in oxygen consumption of rabbits (during July it is 25 to 40 per cent greater than in October to June) are independent of temperature. This variation disappears following thyroidectomy.

It may be noted in conclusion that there are considerable differences (in some cases as great as those reported for race differences) in the BMR of whites in this country as reported from different states. Thus Coons (178) found the average BMR of 10 normal Oklahoma women aged 17 to 36 years to be 32.6 Cal. per square meter or 13.2 per cent below the Du Bois, or 10.1 per cent below the Harris-Benedict and Dreyer standards. Overweight women, aged 17–28 years, tend to have a BMR above normal, while the underweight are below normal. Remington & Culp (179) measured the BMR of 93 nurses and 40 medical men students in South Carolina, and found them to be below the Aub–Du Bois standard: —6.6 per cent for the women, and —6.1 per cent for the men.

Pregnancy and lactation.—The earlier literature on the energy metabolism of pregnancy was reviewed by Needham (180). A series of very significant papers on metabolism during pregnancy and lactation have appeared from Macy's laboratory. Since, however, this review is confined to energy metabolism, only one paper will be mentioned in this volume, leaving the review of the other papers for the second installment (Volume IV). Shukers, Macy, Donelson, Nims & Hunscher (181) reported a detailed study of the intake of energy, protein, fat, carbohydrate, calcium, and phosphorus at monthly intervals, throughout the complete reproductive cycles of three normal

women. The diets were self-chosen. The milk flow was abundant. The daily caloric intake during lactation was from 30 to 55 per cent above the average intake during pregnancy, with a maximum intake which was more than twice the minimum.

Sandiford, Wheeler & Boothby (182) concluded, from an extensive study of one case, that the increase in heat production due to pregnancy, amounting to 25 per cent, can be accounted for by the added metabolism of the young.

Rowe, Gallivan & Matthews (183) found that the BMR rise during the last 24 weeks of pregnancy is 13 per cent more than is predictable from changes in weight, and after falling during the first three to five weeks following delivery to a value below the normal it rapidly returns to normal. Rowe & Boyd (184) criticized severely the method of computation and the conclusions of Sandiford, Wheeler & Boothby based on deducting the fetal from the maternal weight before computing the surface area of the mother. Rowe *et al.* first determined the excess heat production due to pregnancy, and then compared these values with the fetal area. Rowe & Boyd (184), on the basis of the later method, concluded that the 13 per cent excess in metabolism in the later stage of pregnancy cannot be attributed to the fetus, and it is the result of an unknown mechanism involving some factors other than those of fetal-tissue growth.

Schwartz & Drabkin (185) believe that the increased heat production in pregnancy is due in part to the higher metabolism in the maternal organism brought about by the increased activities of the posterior pituitary and the thyroid glands. Evidence is cited from Seitz (186) to the effect that during pregnancy there is a hypertrophy of the thyroid probably in 70 to 80 per cent of all cases, and from Bockelmann & Scheringer (187) that the blood iodine rises from 15.5 γ per cent in the second month to 22.5 γ per cent during the tenth month. That there is an increase in pituitary activity during pregnancy is proven by the fact that there is a tremendous increase in the concentration of pituitary hormones in urine during pregnancy (188). As regards the influence of the pituitary on metabolism, Foster & Smith have shown that this is reduced by 35 per cent after the removal of the pituitary gland, and may be restored to normal by transplantations. Eufinger, Wiesbader & Focsaneau (189) substantiated the idea that there is a hyperfunction of the thyroid during pregnancy. The Reid Hunt acetonitrile reaction was used as a test for the activity of the thyroid. These investigators found that the

resistance of the white mouse to acetonitrile is markedly increased by previous subcutaneous injections of serum from a pregnant woman, leading to the conclusion that this is due to a hyperfunction of her thyroid during this period. Grab (190) likewise found that the administration of anterior pituitary substance to dogs increases the concentration of the thyroid principles in the blood as indicated by the acetonitrile test. This is also shown by increased concentration of iodine in the blood, and by histological changes in the thyroid.

Pommerenke, Haney & Meek (191) concluded that during pregnancy in the rabbit there is an increase in energy metabolism from a normal of 2.61 Cal. per kilogram per hour to 3.19, or from 32.73 to 41.50 Cal. per square meter per hour in the last day of the gestation period.

Benedict & Ritzman (147) measured the metabolism of sheep during pregnancy. They found no increase in metabolism per unit weight or per unit area. Like Rowe, these authors found a decline in metabolism during the first to the third months of pregnancy after which it increases again. Lines & Peirce (148) found that in sheep pregnancy did not appear to increase materially the metabolism up to the time when 3/5 of the normal term had elapsed. The pregnant ewes did not have a materially different rate from dry ewes under the same nutritive conditions. Young ewes two or three months pregnant and on maintenance diets showed about the same heat output (1,080 Cal. per sq. m. per 24 hours) as ewe weaners on autumn pasture.

Brody and associates (109, 136) measured the oxygen consumption during pregnancy and lactation in dairy cattle, beef cattle, sheep, swine, and horses. They found that following the middle of the period of gestation the heat production rises at a greater percentage rate than the body weight. Thus in the case of Holstein cattle (giving birth to the largest calves), the heat production rose to about 39 per cent above the basal level, while the weight rose to 19 per cent above the basal level; in Jersey cattle the rise in BMR was 23 per cent, while the rise in weight was 11 per cent; in sheep, 24 per cent in BMR and 19 per cent in weight; in swine, 22 per cent in BMR and only 4 per cent in weight. Several of the curves indicate a decline in heat production below the basal level in the early stages of gestation. This is cited in partial confirmation of Rowe's conclusions to this effect. There are, however, many exceptions. When the heat increments of gestation are divided by the fetal weight or surface areas,

the results appear to be much too high for the conclusion that the heat increment of gestation represents solely the metabolism of the fetus. It appears that the metabolism of the fetus, especially in the later stages, represents only 50–70 per cent of the total heat increment of gestation. It is not possible to measure the basal metabolism of lactating farm animals. The "resting" metabolism (including the SDA) however was measured, and in the case of dairy cattle it was found to be about 60 per cent above the non-lactating resting-metabolism level; but the food intake of the lactating animals was nearly double that of non-lactating animals. The other species showed rises in food intake of the same order of magnitude, or often higher (100 per cent above the non-lactating level).

Influence of nutritive conditions on BMR.—Gudjonsson (192) found that the basal level of metabolism in rats may be kept at a lower level by reducing the vitamin intake below a certain level.

Wilson, Levine & Kelly (193) found that the respiratory exchange is essentially normal in every respect in cases of infantile marasmus. However, infants in poor nutrition cry and are otherwise active for longer periods of the day than subjects in good nutrition. This prolonged activity may render an excessive fraction of the ingested foodstuffs unavailable for purposes of growth.

Krauss & Kuppers (194) discussed extensively the problem of the effect of nutritional state ("luxus consumption" of Grafe, "secondary nutritional action" of Rubner) on the BMR and on SDA. Complete or partial inanition leads to a lowering of BMR by 25 per cent, which persists for 14 days despite good intake of food. Previous overfeeding on mixed diets caused an increase of over 20 per cent, which persists even if the food is withheld. But overfeeding with carbohydrates or fat has little effect on the BMR unless a subject is on a normal diet for a period of 14 days prior to the measurements; the normal limits lie between +20 and —25 per cent of the standard.

Wiley & Newburgh (195) found no support for the "luxus-consumption" hypothesis of Grafe. They measured the BMR and total energy exchange on a very thin man (28 years, 57.5 kg., 180 cm.), (*a*) while he was on a maintenance diet (3,000 Cal.), and (*b*) when he was overfed (5,000 Cal.). The slight increase in metabolism on *b* could be completely accounted for by the SDA of the extra food. Bonnet (17) found no support for the theory that "luxus consumption" increases the BMR. Evans & Strang (196)

found that when undernourished persons gained 5.8 kilograms (equivalent to 14 per cent of the initial weight, 6 per cent of initial surface) the BMR was increased by 9 per cent, so that the average BMR per unit area increased by 2 per cent. De Bruin (49) found that with one exception the BMR of 15 fat children did not differ from that of normal children. Strang & McClugage (48) found no difference in SDA of a standard meal (40 gm. of protein, 52 gm. of carbohydrates, 26 gm. of fat) in "normal," "obese," and "thin" subjects.

Boothby & Berkhardt (197) measured a subject on four levels of caloric intake: 894, 1,296, 1,620, and 3,353 Cal. per day. The BMR was —20 per cent on the first three levels, when the subject was losing weight, and 17 per cent on the fourth level when the subject was gaining weight. Ingestion of glycine increased the BMR except on the highest caloric level. After muscular work on the highest caloric level, the metabolism fell 10 Cal. below the resting value. This "negative" phase was absent during the three lower levels of caloric intake.

Hetler, Killinger & Plant (198) suggested that the lower BMR in women may be explained in part by the lower protein intake. However, they found no relationship between BMR and protein intake. In this connection it may be noted that Wang and associates (199) found the protein level (varying from 0.6 to 2.0 gm. of protein per kilogram) in the diet did not influence the metabolic rate in normal women, aged 17 to 36 years, even though there was an increase in concentration in the blood of non-protein nitrogen during the high-protein feeding. McClellan, Spencer & Falk (200) measured the calorimetric and respiratory metabolism under basal conditions and after food of two men who lived on an exclusive meat diet for a year. After 6 weeks of the meat diet the BMR was 5–7 per cent above the level on the mixed diet, while at the end of the year it was just below the level at the beginning. The body weight remained nearly constant on a 2,500–2,700 Cal. diet. The R.Q. remained between 0.72 and 0.78 at all times. According to the theoretical values, this would mean the utilization of 34 gm. more carbohydrates per day than was present in the diet. The authors ascribe this to variations in technique rather than to formation of carbohydrates from fat. Lean meat in meals of 300 to 500 gm. induced extra heat from the third to fifth hours inclusive which ranged from 14.6 to 25.0 per cent of the BMR. On one test the increase was 45.3 per cent and the subject

derived 63 per cent of his energy from protein. Wakeham & Hansen (201) found that the BMR of 20 life-time vegetarians was 11 per cent below the Du Bois standard. Six to seven years of vegetarianism is necessary to bring about this effect.

Rubner (202) kept a subject on a low-protein diet for 25 months (33 gm. of protein, 100 gm. of fat, and 300 gm. of carbohydrates daily). The BMR was not affected and nitrogen equilibrium was maintained but at the expense of diminished capacity for muscular work. Uric acid and ash excretion were greatly increased on this diet.

Wesson (203) found that rats maintained on a fat-deficient diet developed a peculiar abnormality, which was corrected by small doses of certain neutral fats. They showed an R.Q. in excess of 1.00 (indicating formation of fat), while rats on a normal diet do not measurably form fat from carbohydrates. The abnormal R.Q. was not produced by increased intestinal fermentation in the abnormal rats and could not be changed by exercise. Wesson & Murrell (204) proved that the effect of ingestion of fat on the metabolism of these abnormal rats was due not to the catabolism of fat, but to the presence in the natural fat of a dietary factor that is concerned with the absorption, storage, or metabolism of carbohydrate, since the amount of the fat ingested was slight and pure ethyl stearate or the "solid fraction" from lard obtained by crystallization from alcohol was not effective. Stunting of growth was not the cause since such animals when given 6.7 mg. of an active fat had normal R.Q.'s. This dietary factor is not found in the unsaponifiable portion of the natural fat and is resistant to moderate treatment. It is not linoleic or linolenic acid which is curative for the Burr syndrome. These results were confirmed by Burr & Beber (205). Wesson & Burr (206) gave a carbohydrate test meal to rats suffering from Burr & Burr's fat-deficiency disease. The R.Q. rose in the first hour above 1.0, indicating a conversion of carbohydrates to fat. The R.Q. of controls rose only to 0.91. The fat so formed afforded no relief to the signs of the disease. This indicates that neither linolic nor linolenic acid is formed by the fats, as these acids are curative. Early in the disease BMR's were above normal, later below normal. Burr & Beber found that the R.Q. of fat-starved rats is very high (1.05) for 12 to 16 hours, and on fasting rapidly falls to the usual level of the R.Q. of fat. In normally fed rats the R.Q. rarely exceeds 1.0 and the decline to the fasting level is much slower.

Influence of drugs on metabolism.—The most spectacular devel-

opment in this field concerns the calorigenic properties of nitrophenols. The contributions came almost simultaneously from four laboratories, namely from Heymans and associates (207), from Magne, Mayer & Plantefol (208), from Cutting & Tainter and associates (209), and from Dodds *et al.* (210). The 2,4-dinitrophenol used by Cutting & Tainter and by Magne, Mayer & Plantefol, when orally administered to humans in doses of 3 mg. per kilogram of body weight, increases the metabolism about tenfold, and may thus be used clinically as a fat-reducing agent; up to 300 mg. (5 grains) per day caused a reduction of 2 to 3 pounds of body weight per day without dietetic restrictions and without unpleasant symptoms (in 100 out of 113 cases). Dodds used 4,6-dinitro-*o*-cresol which is about five times as potent as 2,4-dinitrophenol. Of the 4,6-dinitro-*o*-cresol per kg. of body weight per day 3 mg. raised the BMR in humans 70 to 100 per cent by the third day without a proportionate increase in pulse or body temperature. A single dose is followed by a peak in about 24 hours, then declines to normal in 4 to 5 days. The recommended dose for Dodds's pyretic is 0.5 to 1.0 mg. per kg. of body weight per day. An excessive dose of either pyretic is followed by a rise in body temperature, sweating (secondary to increased metabolism), and possibly a fatal termination. According to Tainter & Cutting, carbohydrates furnish only half of the fuel of the accelerated metabolism; sugar and muscle lactate tend to increase. It is interesting to note that subjects suffering from myxedema are influenced by these pyretics as regards the metabolic increase, but the other symptoms are not relieved.

According to Giaja & Dmitrejevie (211) 2,4-dinitrophenol, as also β-tetrahydronaphthylamine and adrenaline, simply reduce thermoregulation, increasing the sensitiveness of the animal to environmental temperature, so that the body temperature is raised or lowered depending on the environmental temperature. At high temperature (e.g., body temperature) these pyretic substances cause hyperthermia; at low temperatures they cause hypothermia; and at intermediate temperatures they may have no effect. This is in contrast to the toxine of *Pyocyaneus* infection, which does not inhibit the temperature-regulatory apparatus, but only adjusts the body to a higher temperature level regardless of the environmental temperature.

Glucosamine and acetylcholine, on the other hand, reduce the metabolic level. Zummo (212) found that the oral administration of 3–4 gm. of glucosamine to pigeons decreased the oxygen consumption

by 30 per cent, with a return to normal in about two hours. Subcutaneous or intramuscular injections of smaller doses resulted in lowered oxygen consumption, which lasted 4 to 5 hours. Labbe & Rubinstein (213) concluded that acetylcholine is a hormone antagonistic to adrenaline. Its injection lowered the BMR, especially when it was high due to adrenaline injections.

Hackett (214) found that fifteen women who drank coffee daily for breakfast, and occasionally at other times, had an average BMR 6 per cent above fifteen women who had never drunk coffee. The BMR's of both groups were considerably below the Aub–Du Bois and Harris-Benedict standards.

According to Bonnet (17) the SDA of alcohol is equal to the potential energy of the alcohol (i.e., all the energy of alcohol may be recovered under certain conditions in the form of extra heat). Lombroso & Sunzeri (215), on the contrary, reported that only in a limited number of instances is an SDA perceptible for alcohol, and they cast doubt on the theory that the increase in metabolism, when present, depends on the combustion of the substance. Bornstein & Loewy (216), studying the metabolism of alcohol at high altitudes (2,450 m. in the Engadine), found that when taken alone it gives no SDA, but if taken with sugar it is more marked than at sea-level. Nicloux (217) found that alcohol does burn in the body, white mice oxidizing 0.65 mg. per gram of body weight per hour, the rate of combustion being proportional to the weight of the animal. In frogs its speed of combustion is doubled for each 10 degrees increase in temperature.

Kanai (218) found that in rabbits repeated small doses of alcohol given *per os* tend to improve and often quantitatively increase intermediary oxidations. This is demonstrated by the lowering of blood sugar, diminution of the usual hyperglycaemia after glucose or amino-acid administration, rise in liver glycogen, rise in basal metabolic rate (in humans), as well as by the behavior of the C:N, O:N, and O:C ratios in urine. But large doses inhibit intermediary oxidations. Meyer (219) found that the BMR of a heavy drinker was not changed by the ingestion of 240 cc. of alcohol taken either during 2 hours before or on the evening preceding the measurement. The efficiency of muscular work was decreased by 7 per cent when taken 12 hours previously, but not if taken immediately preceding, or 2 hours before the work began. The R.Q. was lowered 0.03 to 0.04, indicating that alcohol is but slowly burned; the speed of its oxi-

dation was not accelerated by muscular work. Atzler & Meyer (220) found that ingestion of 240 cc. of alcohol by a heavy drinker reduced his total performance on a Krogh ergometer by 10 per cent and the rate of performance by 4 per cent if the alcohol was taken 12 hours before work began, but not if taken 4 hours or just before.

In an editorial review on the effect of muscular exercise on the metabolism of alcohol, Carpenter (221) concluded that the majority of investigators believe that their results indicate that alcohol is utilized or burned in muscular work. But the literature does not furnish adequate data to enable one to draw a decisive conclusion as to whether or not muscular activity accelerates the combustion of alcohol. Studies on the metabolism of alcohol should include measurements of the respiratory exchange and the alcohol in the blood, urine, and expired air until the alcohol has disappeared from the tissues. The results and conclusions on the alcohol problem are obviously conflicting and inconclusive.

The influences of drugs related to the hormones are noted in the following sections.

Sex and pituitary hormones.—Brouha & Chevillard (222) reported that intravenous injection of the gonadotropic principle from the urine of pregnant women into male or female rabbits does not change the BMR beyond ± 10 per cent. Kaufmann, Muller & Steuber (223) experimented with three different types of reputedly active ovarian preparations, and found them to be without influence on the BMR of the rat. Arvay (224) found that injection into rats of commercial preparations of oestrogenic hormones from ovaries, placenta, pregnant urine, etc., increases the oxygen consumption by 17.5 per cent. The author believes that the ovarian hormone increases the BMR due to growth of the uterus and to its specific effect upon the internal sex organs. Anterior-lobe preparations had no effect on ovariectomized rats. Verzar & Arvay (225) found a 10–20 per cent increase in BMR of female rats after a combined administration of menformone and theelin. This had no effect on male rats or on spayed females. The calorigenic action is attributed to the increased oxygen consumption of the hypertrophic uterus. Kunde, D'Amour, Carlson & Gustavson (226) found that subcutaneous injections of estrin into adult dogs did not influence their BMR. Udaondo (227) could find no definite relation between basal metabolism and the menstrual cycle. On the other hand, McClendon, Myrick, Conclin

& Wilson (228) found that the premenstrual BMR was about 2 per cent above that of the post-menstrual period.

Verzar & Wahl (229) reported that the hormone of the anterior pituitary raises the BMR of guinea pigs, the rise increasing for a long time by daily injections. This rise is attributed to the discharge of the colloid substance of the thyroid because in addition to the disappearance of the colloid substance there is no such rise after thyroidectomy. Prolan causes no discharge of colloid substance and no rise in BMR. Lee & Gagnon (230) found that subcutaneous administration of the gonad-stimulating principle of the anterior lobe of the pituitary for periods of 8 to 24 days into rats caused no significant change in their BMR. Similar results were obtained with extracts of the fresh gland and with preparations from the urine of pregnant women. The intraperitoneal injections of these materials reduced the BMR in 10 of the 18 rats.

Riddle, Smith & Benedict (231) studied the basal metabolism and the temperature factor in brooding ring doves. Female doves show no change in metabolism due to brooding, either at 20° C. or 30° C. But in the males the metabolism is increased by 10 per cent (after brooding eggs for 10–13 days) when the environmental temperature is 20° C., and by 6 per cent at 30° C., which is in the zone of thermal neutrality.

Himwich & Hynes (232) studied the effects of subcutaneous injections of posterior pituitary extracts (pitressin, pitocin, and "surgical" pituitarin, which is a mixture of these two) on BMR. Pitressin reduces the BMR by 22 per cent, and the depressing effect of pituitarin is due to its containing pitressin. Pitocin increases the BMR by about 15 per cent. Pitressin acts by influencing the "heat centers," thus decreasing the heat loss and diminishing heat production, and by causing certain vascular effects which lead to an insufficient blood supply to the viscera with a resulting diminution in the BMR. The increase due to pitocin is attributed to the extra work the organism is called upon to perform in connection with changes of fat in the liver and blood. In the case of pitressin the magnitude of the change in BMR is dependent on the size of the dose.

Rowe (233) measured the oxygen consumption of circus dwarfs and of an acromegalic giant (height, 3 feet 2 inches to 9 feet 3¾ inches; weight 35.9 to 417 pounds). The dwarfs had the physical stature and sexual immaturity of children, but ranged in age from 18

to 34 years. The BMR was low, in some cases showing deviations of —44 per cent, attributed to abnormalities of the pituitary gland.

Several other papers on hypophyseal deficiency were discussed in the section on SDA, illustrating the fact that SDA is delayed, but does not differ in totality from that in normal individuals.

Thyroid.—Canzanelli & Rapport (234) injected intravenously into dogs tyrosine, diiodotyrosine, diiodothyronine, and thyroxine and noted the effect on BMR. The maximum rise due to 1.0 gm. of tyrosine was 10 per cent of the total metabolism, the effect lasting about 10 hours, the average increase being about 220 Cal. per gram of tyrosine per kilo of animal. Diiodotyrosine produced about 7.5 times as much effect as did tyrosine; diiodothyronine about 15 times that of diiodotyrosine; and thyroxine about 17 times that of diiodothyronine. The greater the total increase the more prolonged, in general, the effect. Excepting thyroxine, the effect of all of the substances began almost at once (½–1 hour); in the case of thyroxine there was a delay of 4 to 5 hours. The data indicate a step-like progression in stimulating influence, as iodine and the phenyl radical are added to the tyrosine molecule in certain positions. The extra heat production after the administration of diiodothyronine, as well as of thyroxine, cannot be used to perform muscular work, thereby putting these substances in the same class with proteins, amino acids, and epinephrin and different from carbohydrates and fats.

Delcourt-Bernard (235) found that small doses of iodine—5 drops of Lugol's solution per day—caused a moderate fall, and diiodotyrosine a rise, in BMR in hyperthyroid subjects. Larger doses of Lugol (30 drops daily) caused a progressive fall. The same author (236) found that the immediate effect of iodine ingestion in a subject suffering from Graves's disease, and having an initial BMR of +55 per cent, was a fall in metabolism after the subject was brought back to normal by operation; then absorption of iodine was followed by a rise in metabolism. Potassium bromide, bromine water, ferric sulphate, and oxygenated water were without effect. He also found that diiodotyrosine given in a case of Graves's disease (BMR, +55 per cent) produced an immediate lowering of the BMR of the same order as obtained with inorganic forms of iodine. Lemort (237) is of the opinion that the favorable action of iodine in hyperthyroidism is not a specific function but a general property of iodine, and that iodine administration will bring about a temporary reduction of metabolism even in normal subjects. Kommerell (238) found that the administration of

0.5 gm. of diiodotyrosine in a dog treated with thyroxine left the increased BMR unchanged.

Gerard & McIntire (239) fed thyroid to dogs for several weeks, then killed them while they showed symptoms of hyperthyroidism. The oxygen consumption of the vagus, liver, and heart tissues was 25 per cent above tissues of normal dogs, while of the thyroid gland it was 30 per cent lower than of thyroids of normal dogs.

Boothby, Buckley & Wilhelmj (240) found that exercise increased the calorigenic action of thyroxine, and this increase persisted for many days after the exercise was stopped. The calorigenic action of 10 mg. of thyroxine averaged 220 Cal. in period 1 (no exercise), 449 Cal. in period 2 (exercise), and 487 Cal. in period 3 (no exercise). A single 10-milligram dose of thyroxine lowered the R.Q.'s to 0.82 to 0.77 for rest, 0.81 to 0.75 for work, and 0.83 to 0.76 for rest following work. The decreases were six times greater than could be accounted for by the increased combustion of fat to meet the extra metabolic needs.

Siebert (241) thyroidectomized 12 guinea pigs as completely as possible. After three weeks 8 animals were given 50 mg. of KI by mouth daily. Four animals were kept as controls. The average BMR in the controls was —18 per cent with an R.Q. of 0.75; in the iodized group it was —32 per cent with an R.Q. of 0.86. Microscopic remains of thyroid tissue were present in most animals. Spence (242) produced simple goiter in 24 rats fed on a vitamin-deficient diet. The BMR of these rats was variable, owing to secondary infections, but they showed significant differences from the controls, indicating a definite thyroid disorder. Four goitrous rats, however, showed a fall in oxygen consumption below normal after nine months. Cole & Womach (243) fed guinea pigs with extracts from patients with toxic goiter, which increased the BMR up to +18 per cent. Lettuce fed to guinea pigs on a balanced diet decreased the BMR, and when desiccated thyroid was fed, it diminished the toxic effect of the latter. Kaiwa (244) found that the BMR of the thyroidectomized and adrenalectomized rabbit is —30 per cent; in order to keep it at the normal level, it is necessary to give it from 0.08 to 0.15 gm. of dried thyroid extract daily. It may be noted in this connection that Franke (245) found the BMR of dogs to be lowered by daily subcutaneous injections of an extract of suprarenal cortex over a period of months.

Marston & Peirce (247) found that thyroidectomy in young

lambs caused a regression of basal metabolism up to —40 per cent, and in such cases typical cretinism was produced. The variability in response to thyroidectomy was traced to the rapid regeneration of thyroid tissue from minute particles left around the base of the thyroid artery at operation. In some cases of older sheep the metabolism was not materially affected after removal of the whole of the thyroid. It may be noted that Marston (246) found that ablation of the pineal gland in the Merino wether caused a marked peripheral dilation, a material increase in energy consumption, increase in body weight, and up to 50 per cent increase in wool growth. Oberdisse & Thaddea (248) found that "antithyroidin-Moebius," prepared from serum of thyroidectomized sheep, depresses the BMR in normal rats, and lowers the increased BMR of thyroxine-injected rats. But the effect is not lasting and the dose for humans would be too high for practical purposes.

Ring, Dworkin & Bacq (249) found that removal of the thoracolumbar sympathetic chains does not prevent the usual rise in metabolism after thyroxine.

Ring & Hampel (250) found that adrenal inactivation (removal of one and denervation of the other), or thyroidectomy, does not lower the high metabolism in depancreatized cats. It is therefore concluded that the high metabolic rate under these conditions cannot be ascribed to the hyperactivity of these glands.

Wyatt, Weymueller & Levine (251) found that infants with apparently normal thyroid glands respond to thyroid extracts, and produce clinical symptoms, as do normal adults. The metabolic response of these subjects to smaller dosages of the drug is inconstant, but the minimum effective dose always exceeds the therapeutic amounts effective in patients with hypothyroidism, which is in agreement with Plummer's rule that the stimulating effect of externally administered thyroid varies inversely with the intrinsic secretion of the organism. The combustion of all three classes of foodstuffs was accelerated to the same extent by thyroid administration.

Meyer, McTiernan & Aub (252) found that thyroxine did not stimulate and sometimes depressed somewhat the metabolism of tumors, while it increased over 20 per cent that of liver tissue.

Means, Lerman & Salter (253) believe that the calorigenic action of whole thyroid gland depends upon its total organic iodine rather than upon its thyroxine-iodine content.

Haney (254) stimulated the nerve supply (cervical sympathetic)

to the thyroid gland with a resulting BMR increase of 22 per cent after five days; there was no such increase if the thyroids were removed immediately after stimulation. Hence it is concluded that such stimulation of the cervical sympathetic results in a secretory effect upon the thyroid gland.

Diabetes.—Rathery, Plantefol & Plantefol (255) concluded on the basis of their respiratory metabolism experiments on diabetic humans that there are several types of diabetes; sometimes the molecule of glucose is not metabolized; sometimes it is attacked in a normal fashion, and sometimes it is so utilized that the R.Q. is lowered.

Muller (256) found a higher oxygen consumption in non-treated diabetic patients. The author believes that the extra oxygen consumption is not a measure of the energy liberated; on the contrary, he believes that the energy metabolism in diabetics is quite normal. A 24-hour study of the R.Q. does not indicate the formation of sugar from fatty acids except during the period when the metabolism is most disturbed.

Topper (257) reported BMR studies on insulin-treated diabetic children. At ages 5–10 years, the BMR was +3 per cent; at 10–12 years +4 per cent; at 12–15 years, +5 per cent; at 15–17 years +21 per cent; over 17 years +7 per cent. The marked increase during 15 to 17 years is a puberty effect. Puberty is delayed in diabetic children as compared to normals of the same race.

Influence of temperature and related factors.—Deighton (114) recently reviewed the literature on the influence of physical factors in body-temperature maintenance and heat elimination. Laurens (258) reviewed the literature on the influence of radiant energy on metabolism.

Kleiber & Winchester (259) introduced the idea of "social temperature regulation" consisting in huddling, thus, in effect, forming a larger biological unit. Chicks below 25° C. decrease heat loss by huddling, thus superimposing social heat regulation over individual regulation. The social regulation is, of course, physical in nature, but it occurs within the temperature limits in which chemical regulation would otherwise take place. At 14°–15° C. the metabolism of chicks decreased in every trial when individuals were allowed to come together. At 37.8° C., however, coming together did not only decrease, but increased somewhat the BMR. These results do not contradict those of Benedict & Fox (126) on mice and of Benedict & Ritzman on sheep (147) that huddling does not affect heat production, because

the later results were obtained above the critical temperature when heat production was above the normal needs.

Giaja & Gelineo (260) investigated the limits of resistance of animals to cold. Liquid air was used to obtain the desired temperatures. The animal gradually loses, step by step, its ability to maintain its body temperature and is finally overcome by the cold. The following are the approximate extreme temperatures at which the animals can just maintain body temperature for a given time: Rat (trained to cold), —25° C.; guinea pig (cavia), above —15° C.; greenfinch (*Ligurina chloris,* L.) —30° C.; sparrow (*Passer domesticus,* L.) above —30° C.; goldfinch (*Carduelis elegans,* Steph.) —30° C.; lark (*Alanda arvenisi,* L.) —40° C.; pigeon (*Columbia livia,* Briss) —85° C.; turtle dove (*Turtor vulgaris,* Eyton) above —45° C.; hen (*Gallus*) above —50° C. Gelineo (261) found that basal metabolism is greatly influenced by the previous environmental temperature. Thus a rat after having been kept some weeks successively at temperatures of 7°, 5°, 18°, and 31° C. had at the end of each period BMR's of 859, 676, and 517 Cal. per sq. m. per 24 hours. The metabolism thus varied by 65 per cent. The adaptation of metabolism to the surrounding temperature can be followed from day to day, being rapid at first and slowing down gradually. The temperature of rats reared at 30°–32° C. falls below normal when they are placed at 24°, while those reared at 16°–20° reach the resistance limit at about 2° C. The thermic environment also influences the "summit metabolism." Thus rats adapted to environments of 30°–32°, 16°–20°, and 0:0 to 2° C., reach "summit-metabolism" values of 746, 1,918, and 3,100 Cal., respectively. ("Summit metabolism" is the metabolism at the lowest environmental temperature at which normal body temperature is maintained.) Giaja (262) fatigued rats by forced running on a wheel. The "summit metabolism" was measured at rest and at different stages of fatigue. Intense fatigue diminished resistance to cold (i.e., the summit metabolism is reduced) by 25 to 30 per cent. Thus at 2° C. the temperature of the rat falls below normal when fatigue becomes intense, whereas the rat in repose maintains its normal temperature in surroundings of the same temperature.

Benedict, Landauer & Fox (263) found that changes in environmental temperature between 15° and 28° C. did not affect the basal metabolism of the normal domestic fowl. But in the frizzles (defective plumage) the metabolism increased about 4 per cent for each degree decrease in environmental temperature from 28° to 15°. The

food consumption of the frizzles is likewise higher, and its physical activity lower than in the normal fowl. While the normal fowl loses 49 per cent of its heat by water evaporation (temperature 17° to 19° and 28° to 30°) the frizzles lose only 17 per cent by vaporization. The surface temperatures of the breast and back of frizzles were nearly 6° higher than those of the normal fowl. At night the metabolism is much lower than during the day. Normal moulting hens had a higher metabolism than normal non-moulting hens. The rectal temperature was nearly the same (41°–42° C.) in all fowls.

Benedict & Fox (126) found that for normal mice the thermally neutral temperature is 28°5, while for hairless mice it is 34°. The BMR was respectively 640 and 750 Cal. per square meter per day. The BMR in normal mice increased 3.6 per cent per degree decline between 28°5 and 15°7; in hairless mice it was 1,000 Cal. per sq.m. at 28°, and 2,250 at 16°.

Swift (264), working in Murlin's laboratory, exposed humans to an environmental temperature of 2° C. and relative humidity of 80 per cent. Shivering increased steadily in vigor and in frequency as the period of exposure continued. The average increase in heat production caused by shivering vigorously 1 per cent of the time was found to be 4 per cent, which is a fourfold increase over basal metabolism for shivering all the time. Actually, shivering being intermittent, the metabolism in any one period rarely exceeds double the basal. Respiration and pulse rates decreased slightly during the exposure. Individual variations to cold are attributed, in part at least, to variations afforded by the insulating body fat. Persons whose weight per unit height (kg./cm.) exceeds 0.45 showed little or no increase in metabolism. The average increase of 11 per cent was observed for the non-shivering periods, probably due to increased muscular tension. The stimulus to shivering cannot be due to fall of internal body temperature as the rectal temperature curves at 24° and 2° C. were parallel. Shivering began, on the contrary, when the skin temperature reached a limiting value (19° C.).

I am indebted to Professor Murlin for assistance in preparing the reviews of the paper by Swift and the following on the influence of electric currents.

Nasset, Bishop, Warren & Peters (265), working in Murlin's laboratory, studied the influence of high body temperature produced by diathermy on the metabolism of anesthetized dogs. The high-frequency apparatus and current used were 10^6 cycles, relatively high

voltage, and 500 to 3,300 milliamperes. The body temperature was raised from 3°.6 to 8°.5 C. above normal. Metabolism increased 27 to 172 per cent. Respiration rate increased up to twenty-five fold. In some cases the metabolism fell after the current had been shut off, even if the body temperature rose or remained constant. Carbohydrates were heavily drawn upon as indicated by a hyperglycemia. If the animal was placed in the high-frequency field (frequency 10^6–10^7 cycles) between condenser plates without contact electrodes, the metabolism increased 24 to 54 per cent per degree rise in temperature. Lung ventilation increased up to fifteen fold. Currents of different frequency gave the same results. The high metabolic rate was accounted for by (*a*) increased body temperature and (*b*) increased activity of respiratory muscles, no influence being attributed to the current as such. The blood-cell volume increased from 2.1 to 10.1 per cent (average 5.3 per cent) per degree rise in temperature. The loss of water from the blood stream was compensated by storage of corpuscles in the spleen. The CO_2 content in the whole blood decreased 2.2 to 8.6 per cent per degree rise in temperature (total rise up to 30 per cent), with an accompanying decline in alkali reserve (6.8–21.1 per cent). The hemoglobin saturation with oxygen was lowered above 41 by 7.37; below 41, the saturation was increased.

Delcourt-Bernard (266) investigated the energy metabolism and its partition between proteins, carbohydrates, and fats in fever produced by vaccine injection. Fat combustion was increased especially in the middle and later periods of the experiment; carbohydrate combustion was increased only in the rigor cases in which it is proportional to the intensity of shivering, but is diminished in non-rigor cases. Protein metabolism was variable. Comparisons with voluntary tremors are given. In cases exhibiting rigor the metabolism tended to diminish before rigor, then rose (112 per cent) during rigor, and remained at approximately 30 per cent after rigor until the temperature began to fall. In cases without rigor the mean rise was 16 per cent. On deducting the extra metabolism due to shivering, the increase in rigor cases was similar to that of the non-rigor cases.

Buschke & Vasarhelyi (267) found that starvation reduces the body temperature in white mice from the normal of 36°–37° C. to 31°–33° after a 24-hour fast, and to 24°–25° after 4–5 days of fasting. If, after being fasted for 4–5 days, the mice were placed in an incubator at 37° C. the body temperature rose promptly (within 1½ hours) to 37°, and declined to the preceding level on removal from

the incubator. Within an hour after administration of food, the temperature returned to normal. Giving 0.5 cc. of milk by mouth or subcutaneously was followed within an hour by a temperature rise of 2°–3°, which returned to the preceding low level in 2–3 hours.

Talbot (268) found the highest skin temperatures over the viscera and lowest on the extremities. Humidity does not affect the skin temperature. Skin temperature declines with decrease in the environmental temperature, the decline being greatest in the extremities, from which the heat loss would be greatest. During brief muscular work, the skin temperature falls, then rises.

As regards diurnal variations in energy metabolism, this was reviewed by Deighton (114), and discussed more recently as it relates to the pigeon by Kayser and associates (269, 157). There is in the pigeon a diurnal variation in BMR of about 15 per cent. The lower BMR at night is assumed to be due to the habitual suppression of excitations due to the visual and auditory paths, because the metabolic rhythm can be changed by change of the habitual diurnal conditions; or it may be decreased or abolished by denervation of the muscles, to the activities of which the diurnal variations are probably due.

Heat regulation and metabolism in poikilotherms (36 snakes, 11 tortoises, 8 lizards, 2 alligators) were discussed extensively (539-page monograph) by Benedict (270). When the body temperature of these animals was raised to 37° C. the metabolism per unit area was found to be 100 Cal. per square meter, as compared to the metabolism of normal mammals, which is of the order of 800 to 1,600 cal. per square meter. The laws governing the heat production of cold-blooded animals at 37° thus appear to be different than in warm-blooded animals. It is concluded that the cold-blooded animal cannot have a high temperature because it has not a sufficient blood supply to the heat-producing tissues.

Muscular exercise (oxidative aspects). — Limitations of space make it impossible to discuss critically the very extensive literature on this problem. Only a few comments are therefore presented.

Steinhaus (271) reviewed extensively the literature on the chronic influences of exercise on metabolism. Simonson (272) described the researches carried out in the Charkow (Russia) Work Laboratory. Atzler (273) recently described the researches in his Kaiser Wilhelm Institut für Arbeitsphysiologie (in Dortmund, Westphalia). The original papers appeared in *Arbeitsphysiologie,* edited by Atzler. The researches of the Laboratoire de Physiologie de Travail (at the Con-

servatoire National des Arts et Metiers, Paris) are being described in the new journal *Le Travail Humaine*. We shall not discuss the papers from these laboratories.

In this country important researches are carried on by the Fatigue Laboratory of Harvard University (Henderson, Dill, Edwards and associates). Dill *et al.* (274) found that at 10,000 feet altitude (Colorado) the oxygen-transport capacity may be decreased by one-fifth, which results in a higher lactic-acid equilibrium. It is suggested that the decreased oxygen-transport capacity is due to a small change in oxygen saturation of the arterial blood upon the limiting value for cardiac output. In this connection, Matthews (275) believes that at high altitudes the rapid respiration necessary to obtain oxygen may dissipate heat as fast as it can be gained by utilizing that oxygen. Consequently there must be a limiting altitude (about 30,000 feet) where human life is impossible without either respiratory aid or artificial heating. Bonnardel & Liberson (276) compared the basal metabolism and energy cost of work at an altitude of 3,457 meters at the Institute International de Jungfraujoch, and at Paris. The basal metabolism was increased 7 per cent; the pulmonary ventilation increased 25 per cent. The efficiency of work at high altitudes was less as there was a 16 per cent increase in excess metabolism due to work.

The effect of external temperature and humidity on physical performance was investigated by Dill *et al.* (277). Humid heat significantly reduces the capacity for supplying oxygen to active tissues and limits work output accordingly. As heat dissipation becomes more difficult, an increasing proportion of the blood is shunted from working muscles to skin, thus becoming unavailable in so far as gas transport is concerned. Breakdown in humid heat is not necessarily associated with much elevation of body temperature, but is commonly associated with a maximal heart rate and other evidence of circulatory failure. The difference in efficiency of work performed in hot (34° C.) and in cold (12° C.) rooms is negligible. Dill and associates (278) found that dry heat (as high as 45° C.) among workmen at Boulder Dam revealed few cases of circulatory failure. During 24 hours in such an environment one may secrete without distress 10 liters of sweat containing 10 to 30 mm. of NaCl per liter. If suitable provision is not made in the diet, salt deficiency terminating in the clinical condition of heat cramps may result. In such cases there was marked dehydration with concentration of serum protein and decrease of serum chlorides (279). Injection of normal saline

brought rapid recovery with retention of water and salt. Heat cramps appear to be due to loss of salt and a disturbance of the normal interionic relationships. The phenomenon of heat cramps is not commonly observed in humid heat, probably owing to the great limitation of work output by circulatory distress before there has been enough sweating to bring about salt deficiency. Vernon & Warner (280) investigated the influence of humidity of the air on capacity for work at high temperatures. The degree of fatigue experienced in dry air was much greater than in moist air of the same wet-bulb temperature. The pulse-rate was somewhat quicker in dry than in moist air. The gross mechanical efficiency fell off slightly between 70° and 100° F.

Dill and associates (281) studied the fuel supply as a limiting factor in work output. The dog was used as an experimental subject, since his oxygen-transport capacity is 2 to 3 times that of humans per unit weight. In one case a dog, unwilling to run more than 5 hours without fuel, ran willingly for 17 hours when sugar was supplied at intervals during the run. In this run of 17 hours his sugar intake was 430 gm. and his energy expenditure 180,000 kg.-m. per kilogram in body weight. Another dog, after a long training period, became capable of long performance without supplementary fuel (282). In one case she completed without food a run of 111 miles, climbing 18 miles, within 27 hours. This run required an energy expenditure of 200,000 kg.-m. and the utilization of much reserve fat, the loss in body weight being 10 per cent. Such an experiment indicates that hard work can be carried on when fat is the principal, probably the exclusive, fuel, for carbohydrates could furnish little more than one-tenth of the energy in such a run which probably is used up within the first few hours. Laird (283) found that, in acceleration of recovery from mental and physical fatigue, milk containing 70 per cent of maltose is superior to milk plus 48 per cent of sucrose, which in turn is superior to milk alone, due to the greater rate of assimilation of maltose. Pampe (284) found that the capacity for work (lifting a weight of 11 kg. 13 times per minute from floor to over head) was not improved by ingestion of 50 grams of cane sugar.

Farkas and associates (285), in Budapest, measured the energy output in the various activities of harvesting by Hungarian peasants and checked the results by food intake and body losses during harvesting. The total energy output of a harvester is of the order of 6,000 Cal. per day. As his food intake is of the order of 4,800 Cal. per day, the laborers lose weight during the harvesting season (about

2 kg. during 10 days). It is shown that a laborer sows daily an area of 4,316 sq. m., binds 288 sheaves, and makes 32 shocks at an energy expense of 1,725 Cal. ($0.4 \times 4{,}316$) for sowing; 518 Cal. (288×1.8) for binding sheaves, and 506 Cal. (32×15.8) for making shocks. Twice as much energy is required for walking on stubble as on smooth soil. The daily expense for walking was found to be 300 Cal.; sharpening and hammering a scythe required 15 Cal.; during the 4-hour period for meals, etc., 420 Cal. were spent; his basal (resting period during the harvesting period) metabolism was 10 per cent above that of the slack period. The total minimum expense is about 5,200 Cal. per day. It is concluded that the work of harvesting (as conducted in Hungary) is one of the severest performed by men. Barnstedt & Kogelschatz (286) recently reported similar measurements in Germany. Christensen (287) found a maximum oxygen absorption of 4.7 liters per minute and a respiration rate of 31 per minute during the performance of severe muscular work (1,680 kg.-m. per min.). The maximum depth of respiration was 3.78 liters. The R.Q. varied from 0.78 at rest to 0.94 during 1,680 kg.-m. per minute of work. Work with the arms was performed less efficiently than with the legs. Schneider (288) found that the limit of physical effort seemed to be due primarily to failure of the heart to augment proportionately to the increased demands of the tissues for oxygen.

McClintock & Paisley (289) measured the extra energy output for walking in 65 children, eleven to fourteen years of age. The average values for cost per horizontal kilogram-meter were found to be 0.6449 cal. for the boys and 0.5949 for girls. The general average for the entire group of boys was approximately 20 per cent higher than the value of 0.538 cal. reported by Smith as the average for 8 normal men. The basal metabolism per square meter of the boys was also 20 per cent higher than for Smith's men, from which it was concluded that the energy cost of walking is directly proportional to the basal metabolic rate.

Swartz (290) published a detailed report of the energy expenditure in certain household tasks. Wang (291) found that the mechanical efficiency varies inversely with percentage of overweight. Procter & Bert (292) found that a high glycogen content in muscle is not an essential factor in efficiency since, while exercise of a leg increased the glycogen content of certain muscles for a time, this was followed by a decrease to the previous normal level.

Benedict & Benedict (293) found that intense mental effort, for

example, multiplying "mentally," increased the average oxygen consumption by less than 4 per cent.

Solandt & Ridout (294) found that recovery (return to basal oxygen consumption) of 3 males in post-absorptive condition from "standing-running" at top speed for 30–45 seconds took place in less than 1.5 hours. The R.Q. of the excess metabolism due to this exercise was above 1.

Fenn (295) found that, while the average sprinter is turning out work at the rate of 2.95 horse-power, the oxygen debt is at the rate of 13 horse-power. The efficiency of this work is therefore 22.7 per cent. In this connection it may be noted that in the reviewer's laboratory (296) it was found that a 4-year-old, 1,500-pound Percheron horse (gelding) pulling loads on a horizontal plane (tread mill) at the rate of 3.1 miles per hour had an over-all efficiency ranging from 17 per cent when pulling 125 pounds to 25 per cent when pulling 375 pounds. The efficiency decreased after this load. At 1.2 miles per hour the efficiency ranged from 12.7 per cent for 125 pounds to 21 per cent for 400 pounds. If the overhead cost of standing is deducted, the efficiency is increased from 21 per cent at 3.1 miles per hour for 125 pounds to 28 per cent for 350 pounds. For the 1.2-mile rate this efficiency increased from 20 per cent for 125 pounds to 28 per cent for a 300-pound load. The efficiencies decreased with increasing weights following these weights. If the cost of walking without a load, as well as of standing, is deducted, then the efficiencies for the 125-pound load are respectively 36 per cent and 33 per cent for a 3.1- and 1.2-mile rate, reaching maxima of 39 and 37 per cent for 225-pound loads, then declining to 33 and 31 per cent for 400-pound loads. Huxdorf (297) from the Pommritz Agricultural Work Experiment Station reported preliminary respiration trials with the object of determining the efficiency of work of horses. The results, presented graphically in terms of cubic centimeters of oxygen per minute, were too preliminary for drawing conclusions except that there were individual differences.

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COLUMBIA, MISSOURI

LIVER AND BILE*

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During the last two years substantial advances have been made in our knowledge of the chemistry of the liver and bile, and older studies have been improved and substantiated. The many and varied phases of hepatic activity are concerned with such diverse types of metabolic changes that it is difficult to give an adequate review of this subject without becoming involved in practically all fields of metabolic study. We have endeavored to include, however, only such material as is of general interest and is specifically pertinent to the liver and bile.

Pigment Metabolism

The part played by the liver in the production of hemoglobin has been developed in an interesting study by Whipple & Robscheit-Robbins. In many previous studies they found that dogs could be standardized with reference to formation of hemoglobin. Repeated bleedings of dogs that were on a standardized diet kept them at the desired stage of secondary anemia, and the hemoglobin content of the blood removed served as an index of the amount of hemoglobin formed. Liver added to the diet greatly enhanced the formation of hemoglobin. On the basis of 100 for the additional hemoglobin formed by a given amount of animal liver added to the diet, livers of man were found to have the following hemoglobin-producing factors: normal, 162; senile and arteriosclerotic, 117; acute infectious, 117; chronic intoxication, 151; passive congestion, 94; amyloid and fatty degeneration, 111; carcinoma, primary and secondary, 75; cirrhosis and hepatitis, 164; and severe parenchymal injury, 48. From these figures it is apparent that the hepatic cell holds tenaciously to its hemoglobin-producing factors. In all of these conditions, if account is taken of the reduced number of hepatic cells per unit of tissue, it would appear that the hemoglobin-producing factors of the hepatic cells are essentially normal. In untreated cases of pernicious anemia, a high value is obtained for the hemoglobin-producing factors and in treated cases values are lower. Aplastic anemia gives high values and in secondary anemia, caused by destruction of blood, the values are above normal.

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Secondary anemia caused by loss of blood gives low normal values, and leukemia also gives low values. The iron content of these livers is varied, and there is definite lack of correlation between iron content and regeneration of hemoglobin. Queen, Hawkins & Whipple found that the spleen and the presence of bile are necessary for normal pigment metabolism. They found that splenectomized dogs could be kept anemic for years, and that animals with bile fistulas could be maintained satisfactorily on the proper diet but that splenectomy was rapidly fatal to these latter animals. After a latent period an enormous overproduction of bile pigment occurs, progressive anemia develops, and death ensues. From these studies it would appear that the body can synthesize the pyrrol aggregate, probably in the liver, as more bile pigment is excreted than can be accounted for by that pigment group in the food. Naegeli & Meythaler expressed the belief that the spleen is largely involved in the stimulation of the liver to form bilirubin. After temporary ligation of the splenic vein, bilirubinemia of the peripheral circulation developed on release of the ligature. The degree of bilirubinemia in the circulation was dependent on the duration of the ligation, and was roughly parallel to the bilirubinemia which had developed in the splenic blood. Interrupting the flow of blood from the spleen to the liver by ligature or anastomosis of the splenic vein to other veins delayed the development of jaundice following administration of toluylenediamine. Itoh found that phenylhydrazine icterus was not affected by splenectomy, but that the icterus was due to retention of bilirubin since the same amount of bilirubin appeared in the blood following hepatectomy. Fiessinger & Gajdos confirmed the observation that bilirubin continues to be formed in the body in the absence of the liver. Rabinowitch studied the bilirubin content of the blood and urine of patients with varying degrees of bilirubinemia. These data definitely indicated that there is no renal threshold for bilirubin, and that amounts of bilirubin now undetectable should be found in normal urine when more sensitive tests are developed. Fowweather has continued his studies of the van den Bergh reaction on various preparations of bilirubin from bile and gallstones. He found that some preparations gave a direct reaction, whereas others gave only an indirect reaction. He suggested that bilirubin formed two separate series of salts which reacted differently, and that the liver converted the indirectly reacting type to the directly reacting type. White found that the residual icterus index of deproteinized serum was proportional to the bilirubin content of normal serum (6:1) or

to the bilirubin content when bilirubin was added to normal serum. The same was true for the indirect reacting serum of hemolytic icterus, but other forms (direct) of jaundice revealed a lower residual index than the amount of bilirubin would indicate.

Carbohydrate Metabolism

The major part played by the liver in determining the character of the dextrose-tolerance curve was demonstrated by Soskin & Allweiss. With constant intravenous injection of glucose in amounts just sufficient to maintain a normal level of blood sugar in hepatectomized dogs, additional single injections of glucose caused delayed disappearance of the injected glucose from the blood (diabetic type of curve). In normal and diabetic dogs that received constant injections of similar amounts of glucose and insulin, the curves were normal with the added single injections of glucose. From these findings it would appear that the muscles are incapable of rapidly removing added sugar from the blood, or that the liver plays some intermediary part in the utilization of glucose by the muscles. In the light of present knowledge, the former hypothesis appears the more tenable. Fiessinger, Palmer & Lançon introduced a new method for removal of the liver without any preliminary operations. Following hepatectomy by this method, there is the usual decrease in the sugar of the blood, and subsequently death occurs from hypoglycemia. However, the administration of glucose has little effect on the survival of these animals after operation. We believe that the response to glucose is a prerequisite for the adequacy of a hepatectomized preparation. The main advantage of preliminary operation to insure adequate return of portal blood is to secure evidence that portal stasis is not present, which is indicated by the survival of the animal after ligation of the portal vein. If the portal return is insufficient the animal does not survive ligation of the portal vein more than a few hours, and the symptoms and subsequent death are due to portal stasis and are not referable to the failure of hepatic function. Many single-stage hepatectomized preparations have adequate portal drainage and respond to administration of glucose, but preliminary assurance is not to be had without preliminary ligation of the portal vein.

The behavior of the glycogen content of the liver under experimental conditions was studied by Murphy & Young, who found that there was some decrease in the glycogen content in the cat anesthetized by chloroform, phenobarbital, di-allyl-barbituric acid (dial), allyliso-

propyl-barbituric acid (numal), sodium secondary butyl-β-bromallyl-barbituric acid (pernocton), chloralose or iso-amylethyl-barbituric acid (amytal). The last-named substance produced the least change in glycogen content of the liver, but it was not possible to take repeated specimens of liver without further lowering its glycogen content. DeGraff, Evans & Vacek found that the recovery of the glycogen content of the liver of the decapitated cat could not be accounted for by the small change in the phosphorus content of the blood or the small amount of epinephrine liberated. Evans, Murphy & Young found that this recovery of glycogen was greater if the liver glycogen was low at the start, and that recovery was not delayed by ligation of the common bile duct or of the hepatic artery, or by ligation of the portal vein. Hall & MacKay produced changes in the glycogen content of the liver by feeding and by administration of glucose and of epinephrine. In normal livers they observed the mitochondria as short bacilliform rods. Livers rich in glycogen contained long filaments, spherules, and plump rods about the nucleus and cell membrane. After animals had been fasted for twelve hours, coarse granules were found without definite arrangement in the cytoplasm. Hall & MacKay concluded that there was some relationship between the mitochondria and the glucose-glycogen equilibrium of the liver. Corkill found that small doses of insulin, sufficient to produce hypoglycemia but not convulsions, would lower the glycogen content of the liver of the Australian opossum. Major & Mann and Major found that small amounts of glycogen could be formed in the liver of depancreatized and phlorhizinized dogs by intravenous administration of glucose. Bodo, Benaglia & Friedman found that in fasted dogs there was an increase in glycogen content of the liver after epinephrine had been injected, but that in well-fed dogs there was a decrease. Gömöri & Marsovszky found that extract of the posterior lobe of the pituitary gland lowered the glycogen content of the livers of white rats.

Deuel found that the glycogen content of the livers of female white rats was less than that of the males, and the fat content was greater. Castration of the male rats did not alter the glycogen content, but ovariectomized female rats gave glycogen values similar to those of the male, and these were lowered by administration of theelin to correspond with the normal female. Deuel, MacKay, Jewel, Gulick & Grunewald found that glycogen in the liver was stored to about the same extent following administration of glucose, galactose, or lactose, but that the glycogen from galactose or lactose was retained longer by

fasting dogs or rats than that formed from glucose. Eckstein administered propionic, butyric, valeric, and caproic acids to rats, and found that propionic acid was the only one to cause glycogen to form in the liver. Stöhr & Henze found some increase in liver glycogen following feeding of aceto-acetic acid. Kapeller-Adler & Rubinstein fed a pure fat diet to rats and, under varied conditions, could find no relationship between the fat and carbohydrate contents of the liver. Miller & Lewis, and Magendantz found no formation of glycogen from *d*-xylose fed to white rats, and Silberman & Lewis observed none following feeding of *l*-rhamnose.

Althausen & Thoenes found that the liver glycogen of rabbits was reduced by fasting and by administration of thyroxine, but that the animals that had received thyroxine were more susceptible to the glycogenolytic stimulus of epinephrine. In phosphorus poisoning the first evidence of functional deficiency was failure of the liver to maintain a normal content of blood sugar. Later, progressive decrease in sugar tolerance was noted, due to lack of glycogen in the liver. With marked hepatic injury, hypoglycemia was produced. Hyperglycemia from epinephrine was increased, reduced, and abolished in the successive stages. Similar observations were made on rabbits following administration of chloroform. The administration of glucose and insulin to normal rabbits decreased a high glycogen content of the liver, and increased a low glycogen content, and the administration of glucose without insulin gave a greater increase in glycogen. Following hepatic injury by administration of phosphorus, chloroform, or manganese, the low glycogen content of the liver was further decreased by administration of glucose and insulin.

Fat Metabolism

The position of the liver in the various phases of fat metabolism is the subject of increasing numbers of studies. Himwich, Chambers, Hunter & Spiers studied the fat in the blood going to and returning from the various organs. They found that the liver removed fat from the blood of the fasting dog and from phlorhizinized dogs up to the fifth day, when fat began to be liberated from the liver. In pancreatectomized dogs the liver added fat to the blood, apparently because of the more rapid mobilization of fat in these animals. Goldfarb & Himwich, using the same technic, demonstrated that the liver adds acetone bodies to the blood of diabetic dogs. Dible showed that the degree of fatty infiltration of the liver of starved rats was dependent

on the quantity of fat available for mobilization from the body depots. Once the mobilization of fat is established, this is the determining factor and it is independent of time. At the end of starvation the percentage of fat in the body falls below that of the liver. Rosenfeld found that the deposition of fat in the livers of dogs, given 30 gm. of sesame oil per kilogram of body weight for four days, would amount to as much as 36 per cent of fat in the liver. This deposition could be prevented by the ingestion of 7 or 8 gm. of glucose or sucrose per kilogram. Scheff & Horner found that diphtheria markedly decreased the fat content of the liver. Paratyphoid infection had no effect, but trypanosomiasis or phosphorus poisoning caused a loss of glycogen in the liver and an increase in the fat. The phosphatid and cholesterol contents of the liver remained unchanged. Loewy & Cronheim studied the fat and phosphatid contents of the livers of guinea pigs, rabbits, chickens, geese, and calves under various experimental conditions such as reduced atmospheric pressure, fasting, phosphorus-, and chloroform-poisoning. In phosphorus poisoning the phosphatid content of the liver increased with the neutral fat. Fatty livers could be developed without change in the phosphatid fraction. Artom found that 14 per cent of the iodized fat was present in the liver one hour after intravenous injection, and only 1.8 per cent remained in the liver fourteen hours later, whereas the iodized fatty acids of the blood rose to their greatest heights. In another study Artom found that, following feeding of neutral fat to dogs, the fatty acids, the total chloroform-soluble phosphorus, the acetone-insoluble phosphorus, and the phospho-aminolipins increase up to the sixth or seventh hour of digestion. By the twelfth hour, the value of these substances in the liver is again normal for fasting animals or for animals on a nonfatty diet. The total amount of fat fixed by the liver is only a small portion of that given. Fränkel & Bielschowsky found performed lignoceryl-sphiginosine in the liver of the hog. They proposed the name "ceramide" for this class of substance, and contended that these substances in the liver give it a major part in fat metabolism and in the synthesis of cerebrosides and diaminophosphatids.

Blatherwick, Medlar, Bradshaw, Post & Sawyer found that fatty livers developed in rats to which liver was fed. The liver that was used for feeding contained a large amount of cholesterol esters and fatty acids but not an increased lecithin content. The addition of carbohydrate, desiccated thyroid extract, or lecithin did not markedly affect this condition. Fatty livers could also be produced by diets

containing 1.0 per cent of cholesterol added to diets which did not in themselves produce the fatty livers. The fatty livers also developed in rats fed cooked whole eggs or cooked egg yolks. Best & Hershey confirmed the observation that lecithin, added to the diet of depancreatized dogs, alleviates the condition characterized by signs of hepatic failure (fatty liver). The action of lecithin is not due to vitamins A, B, C, or D. The addition of fairly saturated fats to the diet hastens the formation of the fatty liver and the critical condition. With Huntsman, they extended these observations to include rats given a fat diet; 2.5 gm. of fat (iodine number 40) administered daily for three weeks produced livers containing a large amount of fatty acids (iodine number 100). The addition of egg-yolk lecithin or beef-liver lecithin to this diet prevented the formation of fatty livers which then contained normal fatty acids (iodine number 132). Addition of choline to the diet also prevented deposition of fat, but addition of glycerol-phosphates, sodium oleate, or amino-ethyl alcohol were ineffective. Best & Ridout found that the addition of 100 mg. of cholesterol to a diet of 20 per cent fat (iodine number 65) would also produce a fatty liver in the white rat. The addition of choline or betaine to this diet prevented the deposition of fat in the liver, but larger amounts of choline or betaine were required than were necessary to prevent the development of a fatty liver from a diet of 40 per cent beef tallow with no added cholesterol. Chanutin & Ludewig found that fatty livers developed in rats fed 2.5 per cent cholesterol in the diet. Diets high in fat inhibited the deposition of cholesterol in the liver, whereas more cholesterol was deposited with a diet high in carbohydrates.

The Detoxicating Function of the Liver

Under this heading we have included a miscellaneous group of subjects, which perhaps should be classified as the normal and abnormal metabolism of normal and abnormal substances by which means the liver maintains normal body function. Barbour & Aydelotte continued their study of the liver in heat regulation and water exchange. They found that the blood was concentrated during the fever produced by cooling the brain, and diluted when the brain was warmed and the body temperature lowered. With fever of neurogenic origin the liver became hydrated, but, after denervation of the liver, fever produced in the same way was not accompanied by concentration of the blood or hydration of the liver. Wakeman & Morrell added to their studies of yellow fever experimentally produced in monkeys. They found that

bilirubin and retention of dye were the earliest signs of hepatic involvement, and that these became increasingly greater as the disease progressed. MacNider kept dogs in a state of semi-anesthesia with alcohol for twelve hours. These animals gave evidence of increased renal elimination of phenolsulphonphthalein, and increased retention of phenoltetrabromphthalein. Polson found that the galactose-tolerance test was the most satisfactory test to reveal hepatic insufficiency in rabbits that had necrosis of the liver, experimentally produced by administration of shale oil, and he also proposed a glycine-tolerance test which revealed a delay in return to normal of the value for blood urea in the affected animals. Pratt, Vanlandingham, Talley, Nelson & Johnson found that the bromsulphthalein test gave evidence of the presence of injury to the liver after poisoning by chloroform or injury to its circulation. They also noted that injury to the liver by chloroform greatly increased the susceptibility of the animals to anesthesia by sodium pentobarbital.

McClure studied the hydrogen ion concentration of the blood going to and coming from the liver. The blood from the liver had a lower hydrogen ion concentration than the portal or arterial blood, and the difference was greater after cyanide poisoning. The liver brings about these changes mainly by removal of lactic acid from the blood. Bollman & Mann found that regulation of lactic acid was disturbed by complete removal of the liver, but that large amounts of lactic acid could be utilized by the liverless animal. Hahn found the disappearance of injected lactic acid to be the same in normal dogs as in dogs with severe injury to the liver caused by chloroform. Fiessinger, Herbain & Lançon found that the liver removes injected polypeptides from the blood. Traumatic autolysis of tissue liberates polypeptides in the blood; these are not evident in the intact animal but become so after the liver is removed. Puccinelli found that the fall in blood pressure and loss of coagulability did not occur following injection of peptone in dogs in which the hepatic veins were ligated. Ring & Hampel found the usual rise in metabolism following removal of the pancreas from cats with Eck's fistula, or following partial hepatectomy. From these findings they believed that the specific dynamic action of protein or other functions of the liver were not responsible for this increased metabolism.

Quick claimed that the diminished synthesis of hippuric acid is an approximately quantitative measure of injury to the liver of patients with hepatic disease. Nasarjanz found that phosphorus poisoning

diminished the excretion of conjugated glycuronic acids in proportion to the degree of hepatic injury. Bellet, Ravdin, McMillan & Morrison found that the liver played no specific part in the destruction of quinidine, but that its concentration in the blood and its electrocardiographic effects were definitely greater after injury to the liver. Biebl, Essex & Mann found that the perfused liver removed more nicotine from the circulating blood than did equal amounts of muscle, and that liverless animals were more susceptible to nicotine. Thorp, Essex & Mann found that the liver did not inactivate or destroy ephedrine. Bollman & Mann found that, in the dog, total removal of the liver was followed by cessation of formation of allantoin from uric acid. Gordon & Rabinowitch reported a case of yellow atrophy of the liver, in which large amounts of copper (180 mg. of copper per kilogram of liver) had been retained for fifteen years since the patient had last worked with copper.

Reichle and Lehman & Hanzlik, and Radwin & Lederer found no characteristic changes in the livers of rats and other animals following administration of cinchophen. Barbour & Fisk found some toxic necrosis of the liver was produced in rats and dogs by large doses of cinchophen, tolysin (neo-arsphenamine), or salicylates. Weis and Permar & Goehring, and Weir & Comfort have added a number of cases in which cinchophen apparently has caused extensive hepatic injury in man.

Bile

An excellent review of the physiology of and pathological changes in the bile was made by Horsters. Gilman & Cowgill found that the osmotic pressure of bile could be altered by intravenous injection of hypertonic or hypotonic saline solution. It thus appeared that the total concentration of salts in the bile was dependent on the osmotic equilibrium with the circulating fluid, as well as the specific character of the secretion. Still, McBean & Ries found that secretin appears to have a direct action on the hepatic cell in stimulating it to secrete a greater volume of bile with correspondingly decreased salt content. Brewer & Ries found that bile salts would increase the secretory pressure of the liver if given shortly after the maximum secretory pressure was present. Finkelstein & Lipschutz found that administration of bile salts, or of bile salts and oleic acid, increased the flow of bile in man. Kauftheil & Rappaport found that iodosalicylic acid increased the flow of bile, whereas the other halogen derivatives of this com-

pound were ineffective. Leites & Isabolinskaya found that the administration of epinephrine, thyroxine, parathyreokin, or atropine somewhat diminished the flow of bile in dogs with chronic biliary fistulas. Pituitrin A, pituitrin, and ergotamine had no effect, while insulin, and to a lesser extent histamine, increased the flow of bile.

Schindel's experiments did not change the excretion of bile salts following the feeding of succinic acid, fumaric acid, pyruvic acid, or dihydroxyacetone. In dogs with bile fistulas which excreted an increased amount of bile acids following the addition of 200 gm. of meat to the diet, he obtained no increase when the following substances were given: glucosamine, proline, malonic acid, glutamic acid, leucine, glycocoll, or phenylalanine. Asparagine, tryptophane, and alanine gave definite increases in the excretion of bile acids. Ries & Still found that bile salts do not affect the conductivity of nerve fibers, but increase the irritability of the vagal endings or junctions, and decrease the sensitivity of the vasoconstrictor endings. Large doses tended to produce a block in both systems, and the medullary centers seemed more resistant than their peripheral components.

Gallstones of the mixed type (94 per cent cholesterol) were dissolved in bile of dogs by Pickens, Spanner & Bauman because of the low cholesterol content of dog bile, but the same stones were insoluble in dog bile saturated with cholesterol. Spanner & Bauman found that cholesterol was held in solution in bile of man by bile salts, soaps, and phosphatids. Under conditions which enable the gall bladder to absorb bile salts, cholesterol may separate either in the gall bladder or in the wall of the gall bladder, as in cholesterosis of the gall bladder. Andrews, Schoenheimer & Hrdina found that the normal gall bladder absorbed bile salts and cholesterol in the same ratio, but that the infected gall bladder absorbed more bile salts, thus permitting separation of cholesterol. Andrews, Hrdina & Dostal found an increased excretion of unconjugated bile salts from diseased livers, and Dostal & Andrews found the bile salt–cholesterol ratio of bile to be independent of dietary factors.

Takata found that bile acids inhibited the activity of phosphatase on glycerol phosphates and hexose monophosphate. The same observation was made in rabbits with obstructive jaundice. Quagliariello found an enzyme in bile which dehydrogenated stearic acid. Fürth & Scholl found that the solubility of oleic or linoleic acid in bile salts was greatly increased by the addition of lecithin and small amounts of such salts as sodium chloride, potassium chloride, or sodium sulphate. Greaves

& Schmidt found that rats with complete biliary fistulas had a negative calcium and phosphorus balance, and that little if any irradiated ergosterol was absorbed from the intestinal tract. Desoxycholic acid by mouth promoted absorption of vitamin D. The rats with bile fistulas had a much greater need of vitamin D than did normal rats on an ordinary diet, and it appeared likely that vitamin D is excreted in the bile.

Ravdin, Johnston, Austin & Riegel found that, regardless of the concentration of solutions placed in the bile-free gall bladder, the concentration of sodium chloride and sodium bicarbonate of the solution approached that of serum in a few hours. Fluids can be rapidly or slowly absorbed by the mucosa of the gall bladder, which also can excrete water into the gall bladder, but the excretion or absorption of salt is not dependent on the direction of flow of the water. Johnston, Ravdin, Austin & Morrison made similar observations with calcium lactate and calcium chloride in the uninjured gall bladder. Riegel, Ravdin & Johnston found that when a solution of bile salts was introduced into the normal gall bladder, absorption of water, absorption of bile salts, and finally a concentration of the bile salts occurred. Little if any cholesterol was absorbed when a suspension of cholesterol in water was placed in the normal gall bladder. When a mixture of bile salts and cholesterol was added, the solution was concentrated; the bile salts were absorbed, but the cholesterol was not absorbed. The injured gall bladder absorbed bile salts faster than it absorbed water, giving a decrease in the concentration of bile salts but an increase in the cholesterol content of the mixture added to the gall bladder. When hepatic bile was used Riegel, Johnston & Ravdin found that bile pigment was not absorbed by the gall bladder, and cholesterol was neither excreted nor absorbed except by the infected gall bladder. Ravdin, Johnston, Riegel & Wright found that bile from the gall bladder had a higher concentration of base, bile salts, and calcium, and a lower concentration of bicarbonate and chloride, than bile from the liver. The pH of normal bile from the gall bladder was lower than that from the liver. Although the concentrations of the various solutes are different, the osmotic pressures of gall bladder bile, hepatic bile, and serum are essentially the same.

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THE MAYO CLINIC
ROCHESTER, MINNESOTA

THE METABOLISM OF BRAIN AND NERVE*

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Since the last review of this subject was written in 1931, there has appeared Gerard's (1) admirable review on "Nerve Metabolism." This is undoubtedly the most important contribution which has been made to the literature within the last few years, and its scope permits far more adequate treatment of many subjects than is possible within the limits of an article such as the present. In spite, however, of the fact that Gerard's review is little more than a year old, there have been further developments in several directions since its publication. An attempt will be made to deal with these, in logical rather than chronological sequence.

Feng found that, if a frog's nerve is soaked in 0.4 per cent sodium iodoacetate for two hours, it fails to conduct, when stimulated in oxygen, much more quickly than does the control nerve. Such a nerve is also more rapidly asphyxiated in nitrogen. Both these effects are diminished, but not abolished, by the presence of sodium lactate (in the case of the nerve in nitrogen, the "protection" is seen only in a more rapid and complete recovery on the readmission of oxygen). Both the electric response and the heat production gradually fail in the presence of iodoacetate, though the curve of heat production is not altered in form. Feng concludes that frog's nerve is capable of utilising lactic acid by oxidation; that while lactic-acid production is not essential for conduction, it does enable normal nerve to perform long hours of continuous function. Gerard & Meyerhof and Holmes & Gerard had found (the former using frog's nerves, the latter rabbit's) that nerve was unable to remove lactic acid in the presence of oxygen. This was confirmed by Holmes, Gerard & Solomon. On the other hand, Schmitt & Cori have recently stated that, when lactic acid has accumulated in frog's nerve as a result of asphyxia, it is partially removed on the readmission of oxygen, the rate of removal being about 6 mg. per 100 gm. of nerve per hour. Holmes (2) had observed that the white matter of the spinal cord (rabbit) also removed lactic acid, though slowly. Chang & Gerard, meanwhile, have found that the oxygen consump-

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tion of nerve, as well as the action current, is diminished by treatment with iodoacetate, the drug being applied in far smaller concentrations than those used by Feng. The fall in oxygen consumption in the presence of iodoacetate is gradual; when it is clearly manifested, lactate causes an increased oxygen consumption, though no such increase is caused with normal nerve. With dilute (0.005 M) iodoacetate, but not with more concentrated solutions, there is an increased aërobic breakdown of phosphocreatine. If the nerve sheath be split, but not otherwise, this breakdown is prevented by lactate. Neither the phosphocreatine breakdown which occurs during asphyxia nor that brought about by activity is increased by iodoacetate.

It is evident that no simple explanation is forthcoming, at the moment, which will harmonise all these findings. There is evidence, some direct, some indirect, that nerve can, after all, oxidise lactic acid. Iodoacetate, on the other hand, does not cause immediate block of conduction, though its effect on lactic-acid formation is rapid. Schmitt & Cori have found that in an atmosphere of 94 per cent carbon monoxide and 6 per cent oxygen the production of lactic acid is as rapid as in pure hydrogen; yet conduction persists, and prolonged activity causes no extra production of lactic acid. There is thus evidence that conduction does not depend on lactic-acid formation. Holmes, Gerard & Solomon found no increased disappearance of lactic acid or its carbohydrate precursors on stimulation, nor is the R.Q. of activity, as determined by Meyerhof & Schmitt, consistent with the view that the metabolism of activity is purely carbohydrate in nature. Gerard suggests that perhaps normally other substances are burned to provide energy; that iodoacetate in some way interrupts the normal metabolic chain, and thereby turns the oxidising machinery on to lactic acid, at the same time increasing the aërobic phosphocreatine breakdown, and diminishing the power of the nerve for prolonged conduction. It would clearly be of interest to know whether the ammonia production of the nerve is affected by iodoacetate; this, so far, does not seem to have been investigated. Meanwhile, it is well to remember that it is dangerous to assume that iodoacetate is specific in its effect, especially when used in high concentration. It may well affect systems other than those concerned with lactic acid.

Cognate to these experiments on peripheral nerve are those of von Ledebur (1) and Holmes (5), both of whom investigated the action of iodoacetate on the frog's spinal cord. Von Ledebur con-

cluded that iodoacetate, whether injected into the intact animal or perfused through the aorta (in the latter experiments, concentrations of from 0.01 per cent to 0.005 per cent were used), abolished the reflex activity (tested by mechanical stimulation) of the cord, while the excitability of the peripheral nerves remained intact. With the isolated spinal cord, he found that, though oxygen consumption and CO_2 production gradually diminished under the influence of the iodoacetate, reflex excitability disappeared before the gaseous metabolism was noticeably affected. The consumption of glucose from the suspension fluid, on the other hand, ceased with the disappearance of reflex excitability, and therefore, before the respiration fell off. The addition of lactate had a slight and transitory effect only, of increasing the respiration. Holmes employed a perfusion technique similar to that of von Ledebur. He tested for excitability of the spinal cord by observing whether or not the preparation responded by convulsions to an injection of strychnine. He found that lactic-acid formation in the spinal cord was stopped very rapidly by concentrations of iodoacetate of 0.01 per cent or lower, but that strychnine convulsions still occurred even when the lactic acid had reached its minimum level, though, ultimately, they were suppressed. This finding was in contrast to that of von Ledebur, cited above; it was revealed because the time intervals chosen for testing were shorter than those selected by von Ledebur. The phosphocreatine content of the cord was reduced nearly as fast as the lactate content, and it had reached its minimum at a time when responses to strychnine were still manifest. These findings seem to indicate that reflex activity depends immediately neither on lactic-acid formation nor on phosphocreatine breakdown, but on some other process, so far quite obscure. The onset of inexcitability by strychnine was delayed by the addition to the perfusion fluid of 0.5 per cent lactate or by 0.05 per cent methyl glyoxal. This, clearly, recalls the results of Feng and Gerard. In view of the fact that the tracts of the cord lack a connective tissue sheath, and also have a much bigger vascular supply than has peripheral nerve, and so may be more accessible than nerve to perfused iodoacetate, it is possible that the gradual failure of activity observed represented a gradual failure of conduction in the white matter of the cord rather than an effect on the reflex machinery of the cell or synapse. The peripheral nerves, of course, were protected by ligatures from the action of iodoacetate during the period of the experiment. This view, particularly in the light of the partial protective

action of lactate and methyl glyoxal, would harmonise with the results of Feng and Gerard. On the other hand, glycolysis and the oxidation of lactic and pyruvic acids are among the most prominent features of the metabolism of grey matter. To believe that they play no direct part in reflex function is difficult: but almost equally severe difficulties are encountered when an attempt is made to explain the phenomena of anaesthesia or of the "hypoglycaemic response," if it be assumed that lactic-acid formation constitutes the immediate mechanism of the activity of grey matter.

Hill (1) has re-investigated the heat production of nerve, using apparatus of greater sensitivity and precision than any hitherto available. The "initial heat" is now found to be very small—of the order of 2×10^{-6} cal. per gm. per second, at 0° C. and about 8×10^{-6} cal. per gm. per second at 20° C. Only by performing experiments at 0° C., when the nerve processes are very greatly slowed, was it possible to be certain that the initial heat really existed at all. It forms only a very small proportion of the total heat. For the most recent data on this point see Feng & Hill (1).

It has been established that the delayed heat consists of two phases: A, which lasts only a few seconds, and B, which lasts 30 minutes or more and is of much greater total magnitude than A. Further recent work on this point will be found [Hill (3)]. Hill (1) discusses the possibility of the initial heat representing lactic-acid production or phosphagen breakdown (but see Schmitt & Cori; Holmes, Gerard & Solomon; and Chang & Gerard). He is inclined, however, to attribute it rather to a condenser discharge than to any chemical process. This suggestion is more fully developed in "Chemical Wave Transmission in Nerve" [Hill (2)], to which the reader is referred for a very fascinating discussion of the problems of nerve activity. Feng & Hill (2) show that, during steady stimulation, a nerve may produce 40×10^{-6} cal. per gm. per second, an amount of heat which agrees well with the oxygen consumption as determined by Meyerhof & Schmitt and by other workers.

For the past few years, the claim advanced by Winterstein [for recent statement, see Winterstein (1, 2)] that there is a difference between physiological nerve stimulation, *Erregung*, and artificial stimulation, *Reizung*, has given rise to a good deal of controversy. Briefly stated, his claim is that such phenomena as extra oxygen consumption on stimulation, and possibly, also, heat production [Winterstein (3)] are artefacts, due to local effects of the electric stimulus. This

view, naturally, has been combated by a number of workers. The fact that increased oxygen consumption is trivial or absent when a nerve is stimulated reflexly or by mechanical interference with the end organs (thus *Erregung* and not *Reizung* secured) is not an argument which carries much weight, since in such circumstances only a proportion, and probably only a small proportion, of the total fibres of the nerve are active (e.g., stimulation of an end organ could only stimulate some of the sensory fibres, and none of the motor: reflex stimulation could at best only activate the motor fibres in a mixed nerve trunk). Susumu Harashima has repeated Winterstein's original crucial experiment, by which the latter claimed to have shown that a nerve consumed extra oxygen only if stimulated inside the respiration chamber; he finds that the extra oxygen consumption is almost the same for stimulation either inside or outside the chamber. That this was true was verified by continuous observation of electric responses. He found that stronger stimuli had to be applied outside the chamber than inside, if the height of the action potential was to be kept constant. He suggests that in Winterstein's experiments the outside stimuli may often have been inadequate though the inside ones gave full responses.

Schmitt (2) has also attacked the problem, using a volumeter method of measuring oxygen consumption and making simultaneous records of the action potential with the cathode-ray oscillograph. He has performed several experiments designed to test Winterstein's theory. (*a*) Since reversal of the ***direction*** of the stimulating current often leads to diminution of the action potential, even though the ***intensity*** of the stimulus is held constant, such a reversal should affect the size of the action potential but should not affect the amount of the oxygen consumed if the latter depends on local changes rather than on the propagated disturbance. In fact, however, the excess oxygen consumption was found to vary with the size of the action potential.

(*b*) With gradual increase in shock strength, the excess oxygen consumption again varies with the size of the action potential; subliminal stimuli even give rise to a diminution in oxygen consumption.

(*c*) The correspondence between size of action potential and extra oxygen consumption also holds good if the stimulus strength remains constant and its frequency is increased; with sufficiently high frequencies, for instance, there is no excitation and the extra oxygen consumption is then zero. All these results point, consistently,

to the fact that the extra oxygen consumption is due to the propagated disturbance and not to any local effect. When bull frog nerves, in particular, are stimulated at a low frequency there may be no extra oxygen consumption. Schmitt is inclined to associate the metabolic processes with the "after potential" rather than with the "spike" of the action current; at low frequencies the "after potential" is less than at high frequencies. This suggestion has been followed up by Schmitt & Gasser, who have compared the size of the oxygen consumption with that of the "after potential." They find that, when nerves are poisoned with veratrine, the resting oxygen consumption is increased by about 15 per cent [but see Hill (4) and Fromherz]. Such nerves show a greatly increased "after potential" and, when stimulated at a frequency of 2 per second, a detectable increase in oxygen consumption, though normal nerves similarly stimulated show no increase. These facts are interpreted as indicating that the "after potential" is associated particularly with oxidative processes. Hill (4) finds that if a nerve is poisoned with veratrine, asphyxiated, and allowed to recover in oxygen, the heat production is increased 100 to 1,000 times. Fromherz has found that in similar circumstances the "after potential" is enormously prolonged.[1] Preliminary asphyxiation is, however, necessary if these effects are to appear. In another communication Schmitt (1) has compared the oxygen consumption, "stimulation potential," and action potential of nerve. He finds that the course of the oxygen consumption follows most nearly that of the action potential.

A series of papers have appeared during the period under review, dealing with the metabolism both of medullated and of non-medullated nerve. Von Ledebur (2) measured the CO_2 production of the rabbit's sciatic in response to stimulation. The cord having been cut through 7 to 30 hours previously, under ether anaesthesia, the limb was stimulated mechanically 10 to 20 times per minute for 30 to 45 minutes. The efficacy of the stimuli was judged by the muscular response obtained. No increased CO_2 production was observed. It is clear that only some of the afferent fibres of the nerve could, in any case, have been active and that these were stimulated only once in 3 or 6 seconds. No detectable increase in metabolism could, in these circumstances, be expected.

[1] After treatment with curare or strychnine, with subsequent asphyxia, the electric response is abolished. Asphyxia is necessary to produce the effect. The phenomena are ascribed to the effect of asphyxia on permeability.

Winterstein & Fränkel-Conrat have investigated the amount of sugar broken down aërobically by the frog's spinal cord, and by mammalian nerve. They find that the isolated spinal cord (frog), treated with strychnine, breaks down sugar in the suspension fluid, and that this sugar breakdown is increased on stimulation. This was apparent, however, in only a few experiments since the rate of sugar breakdown fell off extremely rapidly. The intercostal nerves of cattle and the sciatics of rabbits, cats, and hamsters were also studied. It is claimed that stimulation, however brought about, and whether direct or reflex, resulted in an increase in the sugar breakdown. This breakdown during rest amounted to some 0.37 mg. per gm. of tissue per hour at 37°, and was increased by 100 per cent on stimulation. The fate of the sugar disappearing is unknown.

Rosenbaum (1), working with splenic nerves of oxen, and using the Winterstein microrespirometer, finds an oxygen consumption of 541.8 cmm. per gm. per hour in blood, and 547.6 cmm. per gm. per hour in Ringer at 37° C.; at room temperature the corresponding values are 65.3 and 63.7 cmm. respectively. For intercostal (medullated) nerve, the values are 33.0 cmm. per gm. per hour at room temperature, and 207.5 cmm. per gm. per hour at 37°. Changing the suspension fluid (if the nerve were suspended in Ringer) during the course of the experiment resulted in a falling off of the oxygen consumption. This does not occur with blood or serum, nor with corpuscles suspended in Ringer, and restoring the original Ringer restores the oxygen uptake. From this it is argued that nerve itself, red cells, and serum contain a diffusible *Atmungstoff*. Addition of glucose causes an increase in the oxygen consumption at 37° (two experiments, only, recorded). Measurements of CO_2 production indicate an R.Q. at 37°, of only 0.335; at room temperature the value is 0.78.

Rosenbaum & Halter investigated this point more fully. They confirmed the figures previously obtained for the CO_2 production of non-medullated nerve. They explain the low R.Q., therefore, by the assumption that a large part of the oxygen consumed is used to combine with hydrogen in connection with "dehydrogenation" processes. They claim that at room temperature non-medullated nerve produces ammonia at about the same speed (3.57 γ per gram per hour) as medullated nerve. At 37° the rate is raised and increases progressively throughout the experiment (8.06 γ per gram in the first hour, 17.53 γ per gram in the seventh hour). This they inter-

pret as depending on increased usage by the tissue of nitrogenous substances, after the exhaustion of the store of nitrogen-free materials. Non-medullated nerve (crab), however, contains a huge carbohydrate store [Holmes (1)]. Tang, Tang & Gerard, using lobster nerve (also non-medullated), find that it contains "phosphagen," which breaks down in asphyxia. Initially there are 5.1 mg. of phosphagen P, and 41 mg. of total soluble P (of which 12 are organic), per 100 gm. of tissue. Rosenbaum (2) claims to have confirmed his previous observation that the oxygen consumption of non-medullated nerve is increased by the addition of glucose. The effect is apparent only in the second and subsequent hours after glucose addition, when it may be due to exhaustion of the carbohydrate supplies initially present in the tissues. This, however, is unlikely: see above. If the values given are plotted out as graphs it will be seen that the curves have a continuous and increasing upward trend, a circumstance which suggests that bacterial contamination may have played a part in the effect observed. Rosenbaum contrasts his results with those of Sherif & Holmes; their experiments, however, deal with medullated nerve. Rosenbaum also finds that the nerves break down sugar at a rapidly diminishing rate.

Beresina & Feng have investigated the heat production of crab nerve. The initial heat is 58×10^{-6} cal. per gm. per second, or 0.73×10^{-6} cal. per gm. per impulse. It comprises 1.5–2.5 per cent of the total heat. The resting heat production is 0.69 cal. per gm. per hour. Both phases fail together in asphyxia, and the nerve is very sensitive to iodoacetate. Cowan finds that crab's nerve, like medullated nerve, shows a greatly increased action potential on treatment with veratrine. In this case no asphyxia is needed for the development of the effect.

Prager has found that at room temperature the oxygen consumption of medullated nerve (intercostal of cattle) is constant so long as the oxygen percentage in the gas mixture is greater than 15–18 per cent. Root finds a steady increase in the oxygen consumption of the lateral line nerve of the dogfish after isolation. The CO_2 production remains constant, so that there is a fall in R.Q. and a large increase in CO_2-binding power. Halter has investigated the ammonia production of mammalian nerve, and has reached conclusions of considerable importance. The technique adopted permitted the ammonia production of a rabbit's sciatic to be investigated while the nerve still retained its central connections with the spinal cord,

the cord itself having been transected under anaesthesia some time previously. He finds that, while the nerve retains its connection with the cord and for the first hour, the ammonia production is about 13 γ per gm. per hour. When the connections with the cord are severed, this value falls to 4–5 γ. It is also reduced, though to a less extent, by anaesthetising the whole animal, and is increased by reflex stimulation. When the nerve is fully isolated the ammonia production falls. This is interpreted as being due to the fact that "tonic" impulses are cut off. It can be increased on stimulation, and is reduced by narcotics and by asphyxia. It also shows a progressive fall when the nerve is left at rest, and this fall is ascribed to some process which is associated with degeneration. Tying the common iliac artery and so depriving the nerve of blood supply does not markedly affect the rate of ammonia production. Unfortunately the author omits to give full details of the methods of stimulation employed.

Winterstein (4) has reviewed this and cognate work by his pupils and to this review the reader is referred for a fuller discussion.

Some work may be mentioned at this point which deals with the enzyme systems of nerve. Schmitt & Skow find that nerve contains a small amount of catalase, the "catalase quotient" being 35. (That of mammalian brain is given as 50; and of liver 7,000 [Quotation].) The enzyme is inhibited by very dilute HCN. Its activity is unimpaired by asphyxiation, so that it is unlikely that H_2O_2 can play a part in the "oxidizing reserve" of nerve. They claim also that nerve contains a thermostable substance which catalyses the oxidation of unsaturated fatty acids, such as linolic. Cohen & Gerard have investigated the behaviour of nerve to certain artificial hydrogen acceptors. Only those which yield large amounts of energy on reduction are capable of restoring the power of conduction (in the absence of oxygen) to a nerve which has been asphyxiated in nitrogen. Substances yielding a positive result in these experiments are *m*-dinitrobenzene, iodosobenzoic acid, sodium peroxide, and hydrogen peroxide. Cystine, glutathione, and methylene blue, though they will accept hydrogen, will not restore function. The conclusion drawn is that asphyxial block is the result of energy deprivation: and it is clear, therefore, that the energy yielded by reduction of the hydrogen acceptor, whether it be molecular oxygen or some other substance, is of paramount importance. [For a further discussion of this important point see Gerard (1), p. 535, and Gerard (2).]

An increasing amount of attention is claimed by the problems

presented by the metabolism of brain, as opposed to that of nerve. Stare & Elvehjem find that the oxygen uptake (*in vitro*) of chicken's brain is not affected by vitamin A or B deficiency, except that that of the cerebellum is probably diminished by B deficiency. This stands in contrast to the previous results, obtained, however, on pigeon's brain, by workers of the Oxford School. Sinclair finds that the R.Q. of the minced brain tissue of polyneuritic pigeons in the presence of 0.033 *M* lactate is 0.58 to 0.78, as against 0.88 for normal birds. Himwich, Fazikas, Rakieten & Sanders find similar low figures for vitamin-deficient pigeons and dogs. On adding crystalline vitamin B, the value for polyneuritic brains rises to 0.76 to 1.0, the R.Q. of the extra metabolism being 1.7. Meiklejohn has found that, though addition of small quantities of Vitamin B_1 to the minced cerebral tissue of polyneuritic pigeons (*in vitro*) causes an increased oxygen uptake in the presence of lactate, yet no increase in the amount of lactate removed can be detected by chemical methods. He finds himself faced by the same problem as were Ashford & Holmes (2), namely, that from the tissue as a whole more lactate disappears than can be accounted for by the oxygen consumed. Meiklejohn, Passmore & Peters (1) find that, although normal pigeons' brains show a large oxygen uptake with pyruvate, the brains of avitaminous pigeons show a low one, which, unlike the uptake with lactate, is not increased by giving B_1 [see also Meiklejohn, Passmore & Peters (2)]. In a further paper Gravilescu, Meiklejohn, Passmore & Peters claim that the B_1 effect is specific for the lactate system; succinic oxidation is not affected. Simola finds that extracts of the brains of B-deficient rats contain less "CO-carboxylase" than do extracts of normal ones, though the "CO-zymase" content is not altered in B deficiency.

Peters & Sinclair have investigated the effect on the respiration of brain *in vitro* of various substances which, according to Embden's theory (see Meyerhof) are concerned in the lactic-acid metabolism of muscle. α- (but not β-) glycerophosphate, added to lactate, gives rise to an increased oxygen consumption, and lactate + pyrophosphate gives rise to a larger uptake than lactate alone; this increased uptake is partly fluoride-sensitive. Space does not permit an adequate discussion of this paper.

Gerard & Schachter have compared the glucose consumption of brain and muscle, by determining the glucose content of arterial blood, and of that from the saggital sinus and from the femoral vein

respectively. The rate of glucose consumption falls markedly under the action of insulin and the values throughout compare well with the known figures for oxygen consumption. Before insulin is given, the difference in glucose content between arterial blood and that from the superior saggital sinus is in the neighbourhood of 40 to 50 mg. per 100 cc. In insulin hypoglycaemia it falls to 12 to 20 mg. per 100 cc.

Himwich & Nahum have attacked the difficult problem of determining the oxygen consumption and R.Q. of the intact brain.[2] They have performed experiments both with and without anaesthesia, and upon animals under the influence of phlorhizin, insulin, adrenalin, and pituitrin as well as upon normal and depancreatised ones. Dogs were used in all experiments. Except in the case of adrenalin and pitressin the R.Q. was in all cases unity no matter what might be the R.Q. of the whole animal. Thus they conclude that carbohydrate is constantly oxidised by the brain. They find that dog's brain consumes on the average 14.0 cc. of oxygen per 100 gm. per minute. Himwich, Rakieten, Nahum, Du Bois & Sanders find that the R.Q. of dog's brain *in situ* is reduced by injections of methylene blue. Himwich, Fazikas, Rakieten & Sanders show that methylene blue reduces the R.Q. of excised cortex.

London, Kotscheref, Dubinsky & Katzwa, using the *angiostomie methode,* studied the amount of O_2, CO_2, glucose, lactic acid, and glycogen absorbed or retained by the brain, muscles, liver, alimentary canal, adrenals, and kidneys of dogs, before and after exercise. The brain shows the greatest gaseous metabolism, the greatest consumption of glucose, and the smallest production of lactic acid. Exercise has little effect on the metabolism of the brain; sugar and oxygen retention sometimes fall off, probably because of an increased circulation rate. Since there were no determinations of blood flow no absolute figures for oxygen consumption, etc., are available. Haldi (1) has investigated the rate of lactic-acid formation in excised brain, which he finds to be greater than that of kidney, testis, or muscle. The same author (2) has determined the post-mortem rise of lactic acid in brain and kidney after injections of iodoacetic acid. The results were somewhat variable with brain, less variable with kidney, but in most cases an inhibition was found. In two later com-

[2] For an excellent summary of previous work and for data of new experiments, on the effect of mental effort on the metabolism of the whole animal, see Benedict & Benedict.

munications (3) he states that the lactic-acid content of brain, unlike that of blood, kidney, or muscle, is not affected by injections of sodium bicarbonate. Himwich & Goldfarb find that brain does not remove keto acids from blood in phlorhizin diabetes (and therefore, presumably, cannot oxidise them).

In the first review of this series,[3] experiments by Holmes & Sherif were referred to which seemed to establish the fact that the brain depended for its supply of lactic-acid precursor upon the blood stream. The extent of the post-mortem formation of lactic acid in the brains of mice was found to depend upon the blood-sugar level, provided the latter did not exceed certain values, and to correspond at all blood-sugar levels to the amount of carbohydrate to be found in the brain when post-mortem lactic-acid production was prevented by rapid fixation in liquid air. When, on the other hand, post-mortem lactic-acid formation was so prevented the "initial" values for lactic acid were constant except in hypoglycaemia, when they were definitely reduced. Holmes (3) has carried this work further by determining the pH of the mashed brain tissue in different circumstances. He found that the highest (most alkaline) values were obtained in severe hypoglycaemia, and in these circumstances the post-mortem fall was small. Normal and hyperglycaemic brains both gave lower initial values than the hypoglycaemic ones, and the extent of the fall on incubation depended on the blood-sugar level at the time of death, and corresponded roughly with the change in pH to be expected from the production of lactic acid which previous work had shown to occur. While recognising the crudity of the technique available one feels that these results suggest the possibility that there may be an actual shift in the pH of the living brain tissue toward the alkaline side in hypoglycaemia; while, should lactic-acid accumulation ever occur in the living brain (e.g., in anoxaemia or narcosis), there may be a shift in the opposite direction.

There is now abundant evidence that the brain as a whole has a high metabolic rate, and that its metabolic demands are chiefly for carbohydrate; further, the seat of this intense metabolism is the grey matter or, in more precise terms, the metabolic activity of the neurone is centred in the cell, dendrites and synapses, rather than in the nerve fibre. In view, particularly, of the development of current theories of reflex activity (Creed, Denny-Brown, Eccles, Liddell &

[3] *Ann. Rev. Biochem.*, **1**, 487 (1932).

Sherrington) it would obviously be of great interest to know whether this metabolic activity is centred in the cells themselves, or in the non-medullated processes and synapses (though, as has been pointed out earlier in this review, the precise relationship of carbohydrate activity to function is still in doubt). In the posterior root ganglia and their cranial homologues are nerve fibres and nerve cells but, so far as we are aware, no synaptic junctions. It has been found [Holmes (4)] that the oxygen uptake and glycolytic power of the gasserian ganglia of sheep and oxen are low, and of the same order of magnitude as those of nerve or white matter. If, therefore, the cells of the posterior root ganglia are similar in their behaviour to other nerve cells this is evidence that the metabolically active parts of the neurone are the non-medullated processes and the synapses.

Quastel & Wheatley (2) [see also Quastel (1 and 2) have performed a series of experiments upon the basis of which, together with their previous work, they develop an interesting theory of narcosis. Their fundamental experiments are these. If chopped brain tissue be shaken for 2 to 3 hours in a Barcroft apparatus in the presence of air, its own substrates (lactic acid, etc.) will be oxidised, and the rate of oxygen uptake will fall considerably. At this stage, the addition of further substrate (glucose, lactate, succinate, etc.) will largely restore the oxygen uptake. This uptake is depressed if anaesthetics or narcotics (a wide range of such substances has been investigated) have been present from the beginning of the experiment. They demonstrate that the oxidation of glucose and lactate, rather than that of succinate or paraphenylenediamine, is depressed under the conditions of their experiments. Davies & Quastel find that narcotics inhibit the dehydrogenase systems of brain, and that this influence is more strongly exerted when glucose, fructose, galactose, lactate, or pyruvate form the substrates than when succinate or glycerophosphate are acted upon.

In view of these findings Quastel argues that anaesthesia and narcosis consist in the specific depression of the oxidation of these substances at certain centres in the central nervous system. It was previously well known that narcotics in sufficient concentration depressed the power of brain tissue to perform oxidations, and Quastel's theory is clearly attractive. On the other hand, Bülow & Holmes were unable to demonstrate any depression of the oxygen uptake of fresh brain tissue with concentrations of nitrous oxide, acetylene, ethylene, propylene, or ether, which they had found to be capable of

anaesthetizing the whole animal, though higher concentrations of these and other anaesthetics do, as is well known, depress the oxygen consumption. Bülow has confirmed these results and has found (*a*) that if the brain is depleted of substrates by washing, no inhibition occurs when glucose is added in the presence of narcotics, and (*b*) that she cannot confirm Quastel's experiments (performed according to his own technique) with regard to ethylene and propylene, while the inhibition by acetylene continues even after the acetylene mixture has been replaced by pure oxygen. She therefore attributes the inhibition found by Quastel to an irreversible damage brought about by the narcotic. The position, therefore, is at present obscure. If narcotics act by depressing oxidations at certain specific localised points in the brain, it is obvious that the relatively crude experiments which are at present possible may not necessarily throw any light on the question. Robertson & Stewart find that the brains of animals killed shortly after a large dose of alcohol take up *more* oxygen than the brains of normal controls. Quastel & Wheatley (1) have investigated the oxygen consumption of brain in the presence of a number of substrates, using a technique similar to that just described. Glucose, lactate, and pyruvate were oxidised at approximately the same rate. Iodoacetate inhibited the oxidation of glucose, fructose, and mannose. Since fructose is not converted to lactic acid by brain tissue, the mechanism of this effect is not clear. Iodoacetate inhibited partially the uptake of oxygen in the presence of lactate, pyruvate, and glutamate, and did not affect that due to succinate. Succinate was converted to fumarate by the brain, and its oxidation was "spared" by the presence of lactate, which no doubt competed with it for available enzyme. Quastel & Wheatley (3) find that certain amines (phenylethylamine, phenyl-hydroxy-ethylamine, tyramine, indole, isoamylamine, and mescaline) inhibit the oxidation of glucose, lactate, and pyruvate by brain, in concentrations of 0.12 per cent. Certain other amines have no effect. The possible bearing of this on the causation of mental disease is discussed. Ashford has published further evidence that the production of lactic acid from glucose by brain depends upon a mechanism which is distinct from that whereby lactic acid is produced from glycogen, hexose diphosphate, or hexose monophosphate. On the other hand, there is a common enzyme system responsible for the breakdown of glucose and mannose, and another, probably, common to hexose di- and monophosphate. Much less lactic acid is produced from fructose, galactose, or hexose mono-

phosphate than from glucose or mannose. Hexose diphosphate yields more than the monophosphate, but again much less than glucose or mannose.

Haarman (1) finds that there is an anaërobic disappearance of lactate from brain. Space forbids the discussion of a series of other papers by this author on the subject of lactic-acid production [Haarman (2); Haarman & Stratmann (1, 2)]. In general, his findings are compatible with those of Ashford and are discussed by him.

Lundsgaard has published a most interesting paper on the action of phlorhizin on lactic-acid production, a process which the drug inhibits apparently by interfering with the esterification of glucose and glycogen. The general bearings of the paper cannot be discussed here; he finds, however, that the lactic-acid production by brain tissue in the presence of glucose, measured manometrically, is inhibited by 0.02 *M* phlorhizin. The inhibition amounts to 50 per cent in the first 30-minute period, 70 per cent in the next, and 75 per cent in the third. More marked inhibitions were obtained with other tissues. Lundsgaard points out that these experiments throw doubt on the claim of Ashford & Holmes (1) that glucose does not pass through a phosphate-ester stage when converted to lactic acid by brain. The point is clearly a pertinent one. It must, however, be remembered that there is no proof that the only reaction affected by phlorhizin is carbohydrate esterification.

Of obvious importance is the work of Embden and Meyerhof on the chemistry of lactic-acid formation in muscle. (For review, see Meyerhof.) It has been found that glyceric-aldehyde-phosphoric acid plays an intermediate part in this reaction. Mendel *et al.*, on the other hand, found that glyceric aldehyde inhibits the formation of lactic acid from glucose by brain *in vitro;* he claims, further, that this inhibition is reversed by pyruvic acid in concentrations as low as 5×10^{-4} *M*. The writer of this review has verified the fact that lactic-acid formation by muscle extract either from starch, or from glucose + yeast activator, is not inhibited by glyceric aldehyde, while this substance strongly inhibits glycolysis by brain tissue (unpublished); and this seems to him an important argument in favour of the mechanisms, in the two cases, being quite distinct. He has not, personally, been able to observe the effect of pyruvic acid referred to. On the other hand, Bumm, Appel & Conceiro accept Mendel's results and also claim that "coferment-T" and pyruvic acid both increase the anaërobic glycolysis of brain, and that both reverse gly-

ceric-aldehyde inhibition. Kraut & Borkowsky (1) describe the preparation of "coferment-T" from brain. They claim (2) that "coferment-T" prepared from either brain or tumour inhibits the action of phosphatase (prepared from kidney) on calcium-hexose-diphosphate, but that this effect is in turn reversed by fluoride.

Schwartz & Dibold have investigated the ammonia content of rabbit's cortex. On immediate fixation in liquid air, they find 0.10 to 0.23 mg. of NH_3 per 100 gm. of tissue. There is a "traumatic" rise. The amount of ammonia is lowered in insulin hypoglycaemia but it is not then increased by glucose injection, although the hypoglycaemic symptoms are, of course, relieved. In human brain they find an initial content of 0.03 to 0.41 mg. per 100 gm. which rises to 0.9 mg. on standing ("traumatic rise"). Bülow & Holmes do not find that the ammonia content of mouse brain is altered by anaesthesia.

The first attempt (as far as we are aware) at direct determination of the heat production of the central nervous system has been made by Hoffmann, Holzlöhner & Leegaard, who have measured, by Hill's method, the heat production of the frog's spinal cord. They obtain values of 580 to $2{,}324 \times 10^{-6}$ cal. per gm. per second at 21°. (Hill finds a value, for frog nerve, of 8×10^{-6} cal. at the same temperature.) This enormously greater heat production by the central nervous system falls into line with the metabolic studies.

A few papers dealing with inorganic metabolism may be mentioned. Cannavó & Indovina find that prolan administered to rabbits causes a fall of magnesium in certain tissues but a rise in blood and in brain. Hess, Gross, Weinstock & Berliner find that the Ca of brain is reduced in rickets. There is no relationship between blood calcium and brain calcium, or between brain calcium and the appearance of tetany, and brain calcium is not lowered in parathyroid tetany, and is only raised slowly when the rickets are healing.

That this review is crowded and perhaps confused, the writer is only too conscious. In extenuation he can only plead that an enormous number of papers have appeared in the period under review. To summarise what is, after all, itself a summary, is hardly possible. To prophesy is almost as difficult; yet, to the writer himself, it seems that the time cannot be far distant when it will be possible to correlate with metabolic studies the physiological facts elicited by Pavlov and his colleagues, and the studies by the American and English schools of workers on the electrical phenomena of the central nervous system.

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THE BIOCHEMISTRY OF MALIGNANT TISSUE*

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Malignant tissue, like muscle, kidney, or brain, has a distinctive biochemical behaviour. Some of the work defining the origin, nature, or behaviour of malignant tissues published during the last few years is considered in this review. The production of cancer by pure substances of known constitution has been briefly reviewed by Cook (1) and will probably be more thoroughly reviewed in the near future. Woglom (2) and Watson (3) have summarised the recent work on tumour production by tar; Dickens (4), Dodds (5), and Lundsgaard (6) the work on carbohydrate metabolism, and Claude & Murphy (7) that on transmissible fowl tumours.

Experimental Carcinogenesis

The mechanism by means of which coal tar painted on mice produces tumours is still not understood. Maisin & François (8) and Watson (9) have shown that the addition of liver to the diet of mice increased their susceptibility to tar painting. The feeding of liver does not influence the rate of growth of grafted tumours [Woglom (10)]. Watson (11) has shown that the rat, which normally resists the action of coal tar on the skin, develops tumours if it is painted with tar prepared from turpentine, or with such tar and a lipoid extract of rat tissues. By the injection of tar diluted with lard into Plymouth Rock fowls, McIntosh (13) has obtained four tumours transmissible by emulsions of the cells. Of these tumours three have been transmitted by Berkefeld filtrates. Berenblum (12) found that treatment with mustard gas was antagonistic to tar and other carcinogenic agents: it is possible that this antagonism is due to the irritant action of this substance and not to any specific chemical combination. Berenblum (14) and Mansens (15) have obtained epitheliomata in mice by repeated freezing of the skin with carbon-dioxide snow. Combination of this treatment with tar painting inhibited the production of tumours; the effects were not summated.

Investigation by Mayneord of carcinogenic tars and oils [Hieger (16)], by means of fluorescence spectra, led to the detection of the cancer-producing property of 1,2,5,6-dibenzanthracene [Cook, Hieger,

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Kennaway & Mayneord (17)], and to the synthesis of other carcinogenic hydrocarbons, such as 5,6-*cyclo*penteno-1,2-benzanthracene. 1,2,5,6-Dibenzanthracene has been extensively used and has produced hundreds of tumours in mice, rats, and fowls. 5,6-*Cyclo*penteno-1,2-benzanthracene is slightly more active than 1,2,5,6-dibenzanthracene, and 6-*iso*propyl-1,2,5,6-dibenzanthracene is not so active. Burrows (19) and Burrows, Hieger & Kennaway (20) described the production of sarcomata in rats and mice by subcutaneous and intraperitoneal injection of fatty solutions of 1,2,5,6-dibenzanthracene. Tumours have been produced in a similar manner in fowls [Burrows (21), and Peacock (22)]. Lacassagne (23) injected a solution of 1,2,5,6-dibenzanthracene in lard into the testicle of a rabbit; at the site of injection a malignant epithelial tumour appeared which produced numerous metastases in the lungs. Berenblum (24) and Boyland (25) have described methods for producing aqueous colloidal suspensions of 1,2,5,6-dibenzanthracene and injection of such solutions has produced sarcomata in rats and mice.

At the end of 1932, Cook, Hewett & Hieger (26) isolated a pure carcinogenic compound from gas-works pitch. This was proved to be 1, 2-benzpyrene by synthesis, and is the most potent carcinogenic compound yet described. From two tons of pitch 7 gm. of 1,2-benzpyrene were obtained. This compound can also be considered as a derivative of 1,2-benzanthracene.

Cook *et al.* (27) have found that 1,2-benzpyrene and 5,6-*cyclo*penteno-1,2-benzanthracene have some oestrus-producing power. Cook (1) has drawn attention to the similarities in the formulae of cancer-producing compounds and the recently suggested structures of the sterols, bile acids, and sex hormones.

Along with the other hydrocarbons, the carcinogenic agents mentioned above are readily oxidised photochemically by oxygen to substances which inhibit oxidising enzymes such as lactic dehydrogenase [Boyland (28)]. The oxidation product so formed inhibits the respiration and glycolysis of muscle, brain, liver, kidney, spleen, and malignant tumours [Boyland & Boyland (29), Pourbaix (30, 31)]. At the same molecular concentration, oxidised 1,2,5,6-dibenzanthracene is 600 times as effective as sodium arsenite in inhibition of lactic dehydrogenase.

Constituents of Tumours

Bierich, Detzel & Lang (32) found a relatively high phospholipoid and cholesterol-ester content in human tumours and the ratio, phos-

pholipoid: cholesterol ester, was much higher than it is in normal tissues. Bierich & Lang (33) claim that the ratio, lipoid phosphorus: cholesterol ester, in the Jensen rat sarcoma (JRS) increases with the size of the tumour. This phenomenon is similar to that observed by Bloor *et al.* (34), who found that the corpus luteum of the sow had an increased phospholipoid content when in functional activity. Willheim & Fuchs (35) found more cholesterol in tumours than in the surrounding tissue, but Kellner & Lustig (36) claim that the cholesterol content increases as tumours become necrotic.

Edlbacher & Kutscher (37) examined the phosphorus compounds of mouse carcinomata and detected no phosphagen. Buchwald (38) found pyrophosphate and soluble phosphoric esters in rat and mouse tumours. Franks (39) and Boyland (40) in examining mouse and rat tumours (Crocker 180, C 63, JRS, and other sarcomata) found small amounts of phosphagen, considerable quantities of "pyrophosphate" and esterified phosphate, and very large amounts of acid-insoluble phosphorus which is apparently present as nucleic acid.

Sumi & Nakahara (41) found vitamin D to be present and vitamin A absent in four types of tumours (Rous chicken sarcoma, Fujinawa rat sarcoma, Flexner-Jobling rat carcinoma, and C 63). Kögl, Haagen-Smit & Tonnis (42) estimate that human carcinomata contain about twice as much auxin as do normal tissues. Scott (43) examined 98 benign uterine fibromata and found that they contained no more potassium in general than normal tissue. Human tumours (carcinomas, sarcomas, fibromas, myomas) contain chromium to the extent of .00001 to .0025 per cent [Dingwall & Beans (44)].

Tumours contain reducing substances, as measured by iodine titration of deproteinised extracts. They contain glutathione [Kennaway & Hieger (45)] but not free cysteine [Bierich & Kalle (46)]. It had been assumed, after Tunnicliffe (47), that the reduction of iodine (using the nitroprusside reaction for the end-point determination) was due entirely to glutathione. Investigations in two laboratories [Bierich & Rosenbohm (48), Boyland (49)] showed that this was not the case. The glutathione content of malignant tissues is responsible for only about one-third of the total iodine titre. The writer was able to show that two-thirds of the iodine titre could be accounted for by substances which reduce phenol-2,6-dichlorindophenol in acid solution. Glutathione does not reduce this dye in acid solution, and the titration seemed to be specific for ascorbic acid and adrenaline [Birch, Harris & Ray (50)]. Curative tests on scorbutic

guinea-pigs indicate that not more than one-third of the material present in JRS, which reduces phenol-2,6-dichlorindophenol, is ascorbic acid [Harris (51)]. These results show that a part of the reducing power of tumour extracts is due to some as yet unidentified constituent.

Bierich, Lang & Rosenbohm (52) have described a fluorescent lyochrome or flavine pigment in tumours which is probably similar to vitamin B_2. It has an oxidation-reduction potential of —217 mv. at pH 7.2.

In 1929 Kraut & Bumm (53) extracted from malignant tissues, and later from normal organs, a substance which increased the glycolysis of washed kidney. This substance, which they named "coferment-T," was apparently more abundant in malignant and embryonic than in normal cells. Many papers have been published on its properties, but one of the last to appear by Bumm, Appel & Couciro (54) shows that coferment-T is identical with pyruvic acid. Mendel (55) and Dickens & Greville (56) have noticed that the glycolysis of tissues is often increased by pyruvate. That pyruvate occurs in some tumour tissues and can in some circumstances activate tumour glycolysis suggests that this glycolysis employs the same chemical mechanism as muscle [Meyerhof (57)].

Metabolism

The work on carbohydrate metabolism carried out by Dickens & Šimer has established the fact that the respiratory quotient of tumours is well below unity even in the presence of lactate, while with other glycolysing tissues it is 1.0, in agreement with a carbohydrate oxidation. Tumour is thus quite distinct from any other tissue in this respect. The first results (58) were obtained with various carcinomata and sarcomata of mouse and rat in a Ringer-phosphate medium and showed that the low R.Q. was raised by addition of pyruvate, although the rate of respiration was unaffected. Determinations in Ringer-bicarbonate (59) gave a slightly lower R.Q. than in Ringer-phosphate, although the respiration rate was again unaffected. The results of this research indicate that it is the defective respiration rather than high glycolysis that is peculiar to malignant tissues. Dickens & Greville (60) have shown that the ammonia and urea production of respiring JRS tissue is very small and the low R.Q. cannot be due to protein metabolism. The old observations of Fleisch (61) showed tumours (JRS) to be lacking in citrate and suc-

cinic oxidases, and Vietorisz (62) finds rat, mouse, and fowl tumours to be deficient in succinoxidase as compared with other tissues. Barron (63) found succinoxidase in rat tumours but not in Rous sarcoma or the infective myxoma of the rabbit. That the subnormal respiration may be due to lack of *"Atmungsferment"* is suggested by Barron's (64) work on the effect of methylene blue. Methylene blue increases the respiration even when this has been poisoned with cyanide. Friedheim (65) found that pyocyanine, a natural respiration pigment, raised the respiration of mouse (S 37) and human tumours by 50 per cent. The respiration of JRS is unaffected by addition of lactic dehydrogenase coenzyme or of vitamin B_1 [Boyland (66)].

The high glycolysis of malignant tissue resembles that of muscle and other organs in being inhibited by fluoride [Dickens & Šimer (67), Sellei & Jány (68)], by monoiodoacetic acid [Harrison & Mellanby (69), Krebs (70)], and by a pancreatic extract [Harrison & Mellanby (71)]. The latter workers, however, considered that the mechanism of tumour glycolysis must be less complex than that of muscle because the tumours examined could not glycolyse hexose monophosphate, hexose diphosphate, starch, or glycogen.

Franks (39), however, in comparing some of the phosphorus compounds in mouse tumour (Crocker 180) and muscle, found that incubation of tumour caused a large increase in free phosphate, while incubation with glucose caused a much smaller increase in free phosphate and a definite production of acid-soluble organic phosphorus compounds. Willheim & Schmerler (72) found that the glycolysis of the Ehrlich mouse carcinoma is accompanied by a slight decrease in the free phosphate and increase in the easily hydrolysable phosphate. The addition of fluoride reversed these effects. These results, like those of Harrison & Mellanby, were obtained with tumour cells and cannot be compared with results obtained with cell-free muscle extract or yeast juice. In suspensions of cells it is possible that cell permeability plays a more important part than the cellular enzymes in differentiating between added substances. The most marked and certain difference between the glycolysis of tumour and normal tissue, such as muscle and red blood cells, is that the glycolytic activity of tumour is destroyed when the cell is injured by grinding or freezing [Warburg (73), Ronzoni, Glaser & Barr (74)].

Dickens & Greville (75) have found that tumour (JRS) glycolysis may be increased by addition of pyruvate and decreased by

lactate. They (76) have also compared the conversion of glucose and fructose to lactic acid by tissues and obtained rather surprising results. The placenta and outer foetal membrane of mouse embryo cannot attack fructose, but those of the rat can do so. Foetal tissues in general cannot fructolyse. Downes (77) found that the Sugiura rat sarcoma and the Flexner-Jobling rat carcinoma glycolyse fructose at half the rate of glucose; maltose and mannose were glycolysed as readily as glucose. Since then Dickens & Greville (78) have shown that Jensen rat sarcoma and a fowl fibrosarcoma break down glucose at about twice the rate at which they attack fructose, while the Brown-Pearce rabbit tumour and three types of mouse tumours do not attack fructose. On the other hand Dickens & Greville (79) have also shown that the respiration of the Jensen rat sarcoma is higher in the presence of fructose than of glucose; and it is probable that fructose is oxidised without previous conversion to lactic acid. It would be interesting to determine whether tissues poisoned with monoiodoacetic acid can oxidise fructose.

Crabtree's (80) experiments on the metabolism of slices of the Jensen rat sarcoma have shown that the respiration is much more sensitive to the radiations of radium bromide than is the glycolysis. In this, tumour tissue did not differ from testis, spleen, liver, or kidney of the same animal. The metabolism of malignant tissue is no more sensitive to radium than that of normal organs. Crabtree (81) also found that irradiation of succinoxidase produced an irreversible destruction of the enzyme. By combining studies of metabolism with determinations of viability and rate of growth of the tissue on grafting, Crabtree & Cramer (82) have obtained very interesting results. Hydrocyanic acid, which reversibly inhibits the respiration but does not affect glycolysis, does not alter the transplantability even when used in M/20 solution for one hour. Monoiodoacetic acid and sodium fluoride employed at the critical concentrations which produce inhibition of glycolysis damage tumour cells so that they cease to be transplantable. If the inhibition of metabolism is such that it recovers when the agent is removed, then the transplantability remains unaffected. Crabtree & Cramer (82) have also found that anaërobiosis diminishes, while hydrocyanic acid and low temperature (16°) increase, the susceptibility of Jensen rat sarcoma to radium.

These results are in agreement with those of Mellanby (83) who found that acriflavine used in sufficient concentration to inhibit permanently the respiration of rat tumours prevented subsequent growth

of the tissue on inoculation. Avian tumours transmissible by a virus differ from those of mammals in not losing their power of development on inoculation, even after treatment with acriflavine in sufficient concentration to inhibit completely the respiration. This supports the idea that the transmission of avian tumours differs in nature from that of mammalian tumours.

Enzymes.—Waldschmidt-Leitz & McDonald (84) in co-operation with Hueper, Purr, Weil, Schäffner and Köhler have estimated some of the enzymes of the Philadelphia sarcoma and Walker carcinoma of the rat. These tumours contained about the same amounts of pepsin, polypeptidase, dipeptidase, adenylic-acid desaminase, and guanase as does liver. The arginase activity was about one-half that of liver and appeared to be much greater in the more necrotic tumours. The phosphatase activity was high, being almost as great as that of kidney tissue. The catalase activity was low, being of the same order as that of muscle and only one-thousandth that of liver. Similar results for catalase had been obtained by Lewis & Cossman (85).

Apart from arginase all the enzymes examined were more abundant in the more parenchymatous tumours. Somekawa (86) has made estimations of many hydrolytic enzymes in extracts of Rous sarcoma and chicken muscle. Both tissues seem to have about the same amount of most enzymes, but the tumour seemed to contain more arginase and protease. Edlbacher & Merz (87) had found large amounts of arginase in mammalian tumours. Edlbacher & Kutscher (88) have described an active tumour of nucleophosphatase; this is in agreement with some results obtained by Franks (39). They also found a hexosephosphatase but describe it as differing from that present in liver tissue.

The activation of proteolysis by —SH groups, found by Waldschmidt-Leitz & Purr (89), is in agreement with results obtained on tumour proteolysis [as suggested by Waldschmidt-Leitz & Schäffner (90)] by Voegtlin & Maver (91), who found that aëration of tumour (Walker carcinoma or JRS) extract caused oxidation of glutathione and that autolysis or proteolysis of added fibrin was reduced under aërobic conditions. Proteolysis is dependent not only upon the hydrogen-ion concentration but also upon the oxidation-reduction potential. Voegtlin, Maver & Johnson (92) have apparently been able to obtain protein synthesis *in vitro* with JRS with high oxygen concentration in neutral solution. Rondoni & Pozzi (93) found the

auto-proteolysis of liver and Ehrlich mouse carcinoma extracts was increased by addition of cysteine and diminished by H_2O_2. These workers consider that protein synthesis can occur only in the presence of oxygen or other hydrogen acceptors. Possibly the absence of hydrogen acceptors results in the death and autolysis of the cell, although this would not be in agreement with the growth of tissue cultures in pure nitrogen for five days as described by Laser (94). Although the synthesis of complex carbohydrates and proteins appears to involve oxidative processes, it is obvious that these syntheses do take place under conditions of oxygen shortage such as occur in tumours and the anaërobic bacteria.

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THE STRUCTURAL CHEMISTRY OF THE ANIMAL PIGMENTS*

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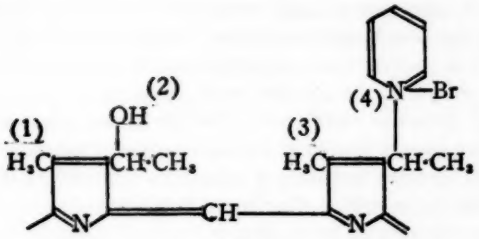
Ferments Containing Haemin

Cytochrome.—During the last two years considerable progress has been made in this field. The investigations, however, were restricted to the component *c*, since this alone, as the most stable component of the cytochrome system, possibly occurs in the native state and can at all events be isolated by catalytic means from the cell. As a result of the far-reaching improvement by Zeile & Reuter (1) in Keilin's method (1930) of isolating cytochrome-*c*, it was no longer difficult to determine the constitution of this substance by chemical methods. Worthy of mention are the preliminary investigations of Hill & Keilin (2). Zeile & Reuter (1), however, first succeeded in isolating crystalline haemato-porphyrin from cytochrome-*c* by HBr-glacial-acetic-acid degradation. They identified its reduction product as a porphyrin, identical with the mesoporphyrinester of blood pigment, thus establishing the structure of the side chain as of the same type as that of blood haemin. A porphyrin soluble in water, with absorption bands lying between those of proto- and haemato- porphyrin, is obtained by the careful splitting off of iron by treatment of an aqueous hydrochloric-acid solution of component *c* with a reducing agent.

According to MacMunn's conception that the histo-haematins are coloured proteids, it would follow that porphyrin-*c* might be considered as a porphyrin in which the linkage with the colloid carrier or its component parts has not yet been broken. This would also provide an explanation of its anomalous solubility properties (hydrophile in character) in comparison with the ordinary porphyrins. Zeile (3) tried to synthesize model structures of porphyrin-*c* and, in fact, succeeded in producing the addition of tertiary ring bases such as pyridine on to the unsaturated side chains of the protoporphyrin. The constitution of the pyridine addition product, obtainable in crystalline form, is shown in Formula I.

* Received January 12, 1934.



I

The position of the pyridine ring in the side chain, whether at 2 or 4, has not yet been determined. As far as one can say today, the structure given in Formula I represents in principle the only method of coupling that explains the properties of the natural component *c*. It can therefore be affirmed, with a fair degree of certainty, that the same structural principle is operative in the natural product.

Another nitrogenous substance besides the one chemically related to porphyrin belongs to the cytochrome-*c* system. It enters into a dissociation equilibrium with iron and is to be considered responsible for the occurrence of the typical haemochromogen spectrum of component *c*. While a differentiation of the nitrogen groups within the system is not yet possible, a delimitation of the cytochrome-*c* system as a whole can be achieved. This possibility was provided by the varying adsorptive behaviour of cytochrome-*c*, depending on its state of oxidation. Under favourable conditions, cytochrome-*c* in the reduced state is not at all or only very slightly adsorbed on kaolin. On the other hand it is easily adsorbed in the oxidised form, in fact without any change of the reaction medium, by merely shaking for a short time with air. Thus it is possible to determine the quantity of accompanying substances in a cytochrome solution in which the pigment occurs together with foreign substances, by carrying out an adsorption in the reduced solution, in which case the cytochrome is not adsorbed. In a parallel experiment the total quantity of cytochrome and accompanying material is obtained by converting the cytochrome into the form in which it is adsorbed, by oxidation in air. The net amount of cytochrome can be calculated from the two results, and thereby the haemin content found spectrophotometrically. This proved to be 3.5 per cent, which gives the minimum molecular weight of cytochrome-*c* as 18700. Hence it follows that cytochrome-*c*

possesses a structure of high molecular weight, as is actually required by the MacMunn conception. A haemin content of 3.5 per cent also indicates the limit of purification by physical methods. The molecular weight in the physical sense, however, as determined from the rate of diffusion represents a multiple of the smallest unit. The position here is very similar to that in the case of haemoglobin, where the haemin content indicates a minimum unit of 17,000 and the actual molecular weight is four times this value.

A few observations by Coulter & Stone (4) should be mentioned that may possibly help toward an explanation of component *a*. The treatment of extracts from *Bacterium diphtherae* and *Bacterium phosphoreocens* with glacial acetic acid, ether, and hydrochloric acid results in the splitting off of a so-called "complex porphyrin" body insoluble in hydrochloric acid with an absorption at 575 mμ and 536 mμ of a porphyrin soluble in hydrochloric acid.

It also shows the change between the oxidised and the reduced stages, and decomposes on treatment with strong hydrochloric acid or slowly with an alkali into a porphyrin complex salt and porphyrin. The recent isolation by Negelein (5) of a haemin from the muscle of the pigeon, as well as the heart muscle of the horse, is also of interest in this connection. It was crystallised out as a pyridine haemochromogen and showed a sharp absorption band at 587 mμ.

Haematoprosthetin.—This designation was given by Herzog (6) to a compound that was obtained from Schalfejew's haemin and other derivatives of the prosthetic group. In contrast to the usual haemin derivatives with 34 carbon atoms, this substance contains only 33 carbon atoms. By boiling with sodium chloride in acetic acid in the presence of methyl alcohol, but not in its absence, haematoprosthetin may be carried over into Teichmann's crystals. In accordance with the opinion of Herzog, not haematin, $C_{34}H_{33}O_5N_4Fe$, but haematoprosthetin should be regarded as the prosthetic group of blood pigments. In agreement with earlier analytical data the author believes that haematoporphyrin, mesoporphyrin, protoporphyrin, and haemoporphyrin contain 33 carbon atoms and only Schalfejew's haemin possesses 34 carbon atoms.

The same author (7) succeeded also in isolating a crystalline CO-haemoglobin, starting on the one hand from human haematoprosthetin and horse globin, and on the other hand from beef haematoprosthetin and horse globin.

Fischer & Zeile (8) were able to show that haematoprosthetin

can be carried over into Teichmann's crystals without the least difficulty. Also the analytical data for the latter indicate the presence of 34 carbon atoms, in agreement with Herzog.

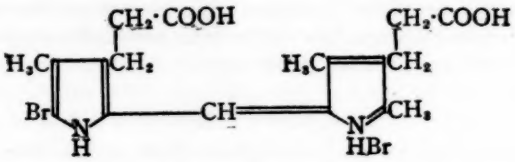
Haemocyanins

To decide whether the haemocyanins from various sources differ in constitution, Hernler & Phillipi (9) carefully examined six different haemocyanins, four of which occur in molluscs and two in arthropods. In spite of their different origins they showed a very remarkable similarity. Thus the copper values of the mollusc haemocyanin, on the one hand, and the arthropod haemocyanin, on the other, are practically the same. Of importance is the fact that the sulphur content is found to be double that of the haemoglobins. Consequently the S : Cu ratio in the haemocyanins is quite different from the S : Fe ratio in the haemoglobins. The nitrogen values show only slight variations, while the carbon values, with one exception, uniformly average 1 per cent lower than the carbon values of the haemoglobins. To explain the difference in copper content between the two varieties of haemocyanin (mollusc and arthropod), Gatterer & Phillipi (10) tested the haemocyanins for the possible presence of other metals and were able to show that no metal other than copper is present.

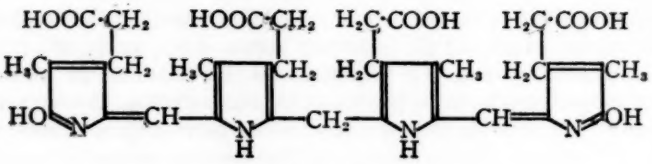
Svedberg & Eriksson (11) have also determined the molecular weight of some haemocyanins with the aid of the ultra-centrifuge. The haemocyanin obtained from *Octopus vulgaris* in which two components, A and B, occur in the region of pH 4.3–9.7 gave by this method a molecular weight of 2,000,000 for component A. That of component B could not be accurately measured, but it is at all events less than that of A.

Bile Pigment

During the last two years a series of experiments have brought new and valuable achievements in this field of research. Starting from bromopyrromethene (II), Fischer & Adler (12) obtained, by treatment with silver- or calcium acetate, an oxypyrromethene in which OH replaced Br. At the same time, however, a somewhat unexpected reaction occurred in which a body "koprobilirubin," very similar to mesobilirubin, was formed by the loss of one carbon atom. Koprobilirubin can be reduced to a leuco compound by the action of sodium amalgam. This compound gives the Ehrlich reaction and

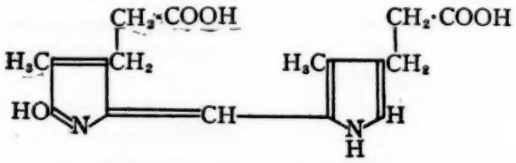


II. Bromo-pyrromethene



III. Koprobilirubin

after a short exposure to light it shows a positive urobilin reaction with zinc acetate. The presence of such a koprobilirubin in animal life, especially in the case of a pathological subject such as in "porphyria," is definitely within the realm of possibility. The similarity between koprobilirubin and mesobilirubin is also very marked in the analogous behaviour in the resorcin melt—a fairly direct reaction produces a pyrromethene of Formula IV.

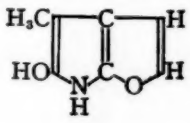


IV. Pyrromethene from koprobilirubin

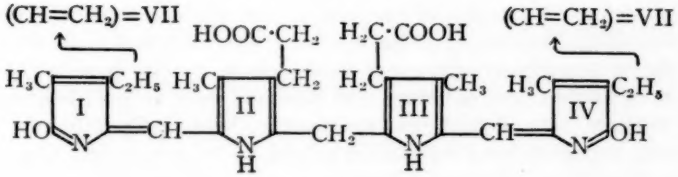
Complete reduction with hydroiodic acid and glacial acetic acid gives a good yield of kryptopyrrol-carboxy acid which is in very good agreement with the results obtained in the reduction of bilirubin. The re-synthesis of koprobilirubin from IV with formaldehyde was at the same time a proof of the correctness of the formula. For purposes of comparison aetiomesobilirubin (13) was examined in con-

nection with its reaction toward the resorcin melt and mild hydroiodic acid reduction. In exactly the same way as with koprobilirubin, the melt provided, as one product of splitting, 4,3′-dimethyl-3,4′-diethyl-5-oxypyrromethene, which with formaldehyde regenerated the original substance. On reduction with hydroiodic acid and glacial acetic acid, bilirubin acid-coupling proceeds, producing on the other hand the analogues to bilirubin acid and neobilirubin acid.

Judging from a series of inconsistencies and a large number of long-known differences in the melting-points of the xanthobilirubin acids, as well as the isolation of haemopyrrol from bilirubin, it was an obvious deduction that bilirubin has a similar side-chain structure as haemin. This view was in the main supported by the isolation of a "nitrite body" of Formula V [Fischer & Hess (14)], which could not be explained by the existing bilirubin formula. On the basis of the assumption hitherto prevailing, bilirubin is thought to be derived



V



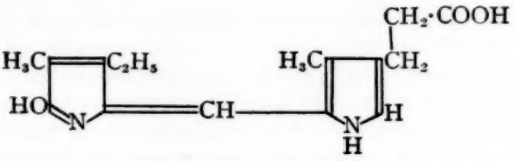
VI. Mesobilirubin [(C_2H_5) in rings I and IV]

VII. Bilirubin [$(CH = CH_2)$ in place of (C_2H_5) in rings I and IV]

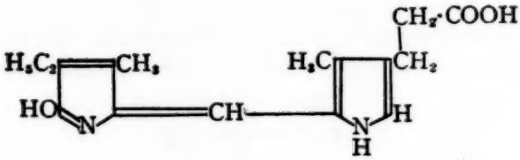
from haemin xiii,[1] whereas by derivation from the natural haemin ix, mesobilirubin or bilirubin should have Formula VI or VII, respectively.

[1] For the nomenclature of the haemins or the porphyrins from which they are derived, compare Fischer & Stangler, *Ann.*, **459**, 62 (1927).

On the basis of the formula above, mesobilirubin should give a mixture of two isomeric neoxanthobilirubin acids (Formulae VIII and IX) in the resorcin melt.



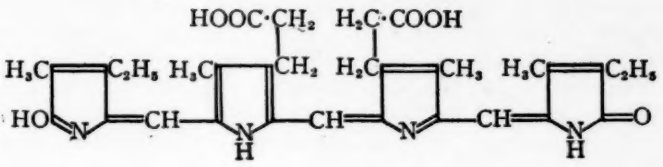
VIII. Neoxanthobilirubin acid



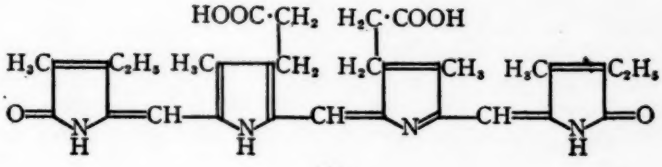
IX. Iso-neoxanthobilirubin acid

That this really does occur was definitely shown by Siedel & Fischer (15) both synthetically and analytically. By a thorough comparison of the melting-points and the mixed melting-points of the synthetic as well as the analytical products of, on the one hand, the free neoxanthobilirubin and *iso*-neoxanthobilirubin acids, and on the other, the condensation products with formaldehyde, it could be shown that mesobilirubin, and therefore also bilirubin, have an unsymmetrical structure. The physiological formation of bilirubin from haemin is thus explained by a mechanism in which the α-methene bridge is oxidised away with the formation of two hydroxy groups on the pyrrol rings I and IV. The assumption of an unsymmetrical structure for mesobilirubin explains all the known facts about bilirubin, and in particular the formation of haemopyrrol by reduction as well as that of the "nitrite body" on oxidation with nitrous acid.

The blue phase that is such a basic characteristic for the Gmelin reaction for bilirubin and mesobilirubin is to be ascribed to the body, "glaucobilin." This compound was thoroughly investigated by Fischer, Baumgartner & Hess (16) and its constitution can be represented by Formula X or XI, wherein the only difference is the position of the double bond.



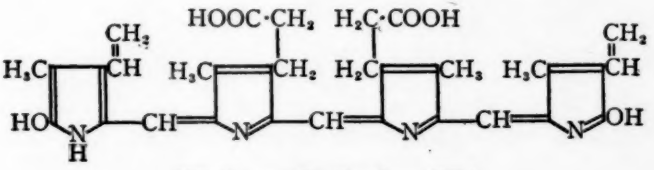
X



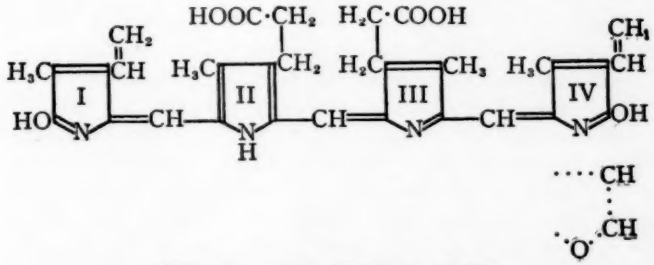
XI

Alternative formulae proposed for glaucobilin

Lemberg (17) was able to isolate uteroverdin from the placenta of the dog and ascribed to it (18) the constitution of a dehydrobilirubin (XII). Siedel & Fischer (15) prefer Formula XIII for



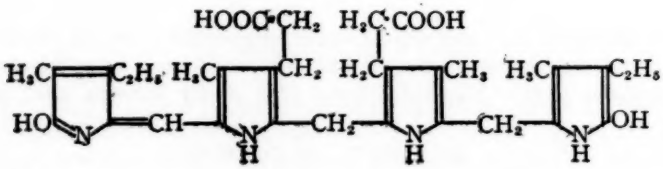
XII. Uteroverdin [Lemberg (18)]



XIII. Uteroverdin (Fischer & Siedel)

uteroverdin or dehydroverdin, but leave it as an open question whether perhaps, as in the case of bilirubin, a dihydrofurane ring must be added to the pyrrol ring IV.

The prolonged catalytic reduction of bilirubin produces mesobilirubinogen as the final product via mesobilirubin. It contains four hydrogen atoms more than mesobilirubin and it may be assumed that an intermediate stage occurs between the mesobilirubin and the mesobilirubinogen. Fischer & Baumgartner (19) succeeded in the isolation of such a substance. This hydrogenation product, the so-called "dihydro-mesobilirubin" (XIV), differs from mesobilirubin partly by its very much higher melting-point (278°) and its violet Ehrlich reaction, but also by the absence of the Gmelin reaction. The fluorescence test is also negative. By the action of sodium amalgam the dihydro-mesobilirubin is further converted to mesobilirubinogen.

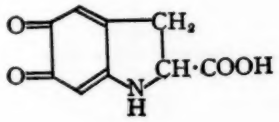


XIV. Dihydro-mesobilirubin

Watson (20) was the first to succeed in isolating stercobilin as well as kopromesobiliviolin and kopronigrin from human excrement. The free, well-crystalline stercobilin as well as the chlorohydrate are of particular interest in this class of substances since there is the possibility of an identity with the urobilin that occurs mainly in pathological urine. It has only recently become possible to obtain crystalline urobilin from urine, and, at the same time, there are definite indications that urobilin and stercobilin are identical (21). There is a definite degree of certainty that both bodies have the formula $C_{33}H_{44}O_8N_4$. While after reduction the Ehrlich reaction still remains positive, no crystalline mesobilirubinogen is formed. Oxidation produces no methyl-ethyl maleinimide. Most probably haematin acid is produced by the CrO_3 oxidation of the stercobilin, previously reduced by amalgam.

Pigment from *Halla parthenopaea costa*

The outer skin of *Halla* contains a red pigment, the constitution of which was determined by Mazza & Stolfi (22) and shown to be a 5,6-quinone of 2,3-dihydroindole-2-carbonic acid (Formula XV).



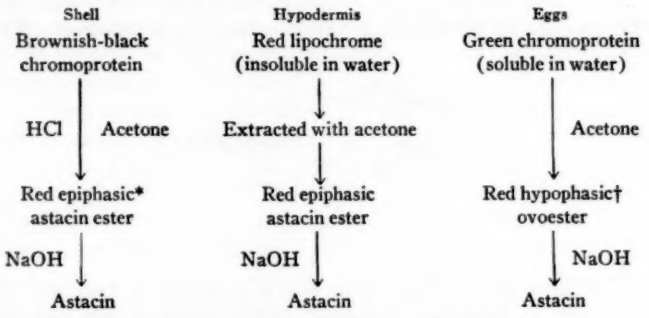
XV

Distillation with sodium hydroxide in a stream of hydrogen produces pyrrolin from this compound, while oxidation with hydrogen peroxide results in a pyrrol tricarboxy acid. The action of sulphur dioxide in aqueous solution gives 5,6-dimethoxyindole and 5,6-dimethoxyindole-5-carboxy acid. Friedheim (23) showed that in the light of its oxidation-reduction behaviour, this pigment plays an active part in the oxygen economy of animals. The leuco body is almost colourless in hydrochloric acid solution, while the colour darkens to an olive brown with increasing pH. In a strongly alkaline medium the pigment soon becomes reversible and is changed into a yellow body. Hallochrome is able, even in concentrations of M/9000, to double the respiration of unfertilised sea-urchin eggs, of fertilised ascaris eggs, and of the red blood corpuscles of the rabbit. It is reduced in the process and can always be regenerated in a stream of oxygen.

The Pigments of Crustaceans

According to Verne (24) the bluish-black pigment of the shell of the lobster should be a protein compound of a red carbohydrate, which could be considered as an isomer of carotene. The pigment of a Scandinavian lobster (*Astacus gammarus*, L.) was subjected to a thorough spectroscopic [compare also Lönneberg & Hellström (25)] as well as chemical examination by Kuhn & Lederer (26). These investigations showed that the various types of pigments of the shell (red with mottled brown), of the hypodermis (orange red), and of the eggs (green or red) can be derived from a parent body, the so-called "astacin," by various esterifications and coup-

lings. The following summary shows the relationships that were found:



* Remaining in benzene (benzene /90% methanol) in the distribution test.
† Remaining in alcohol in the distribution test.

Astacin crystallises from pyridine-water in violet needles with a metallic lustre which decompose at 241°–242°. When pure, it is fairly stable in air and is similar in colour to bixin. Its absorption spectrum in pyridine shows only one apparently homogeneous band at 500 mμ. Astacin contains no nitrogen, has 3 or 4 oxygen atoms, and the composition $C_{27}H_{32}O_3$ or $C_{36}H_{44}O_4$. Hydrogen, to the extent of 10.9 mols, is taken up by catalytic hydrogenation, while a colourless body is produced by reduction with hydroiodic acid. Astacin in alcoholic solution goes into the benzene phase on the addition of water and can again be removed from the latter by dilute sodium hydroxide. This behaviour, as well as the esterification of the hypophasic and epiphasic esters, points to the conclusion that astacin is a carboxy acid. The behaviour on reduction as well as on oxidation with chromic acid, which produces 5 molecules of acetic acid [Kuhn, Lederer & Deutsch (*27*)], has further resulted in the assumption that the astacin molecule must contain 5 dehydrogenated isoprene nuclei, $(=CH-CH=C(CH_3)-CH=)$. Hence it was finally concluded that, in spite of the absorption spectrum of only one band, it is a "carotinoid."

A few other crustacean pigments that also belong to the astacin group must be mentioned. "Vitellorubin" (decomposing at 241°–242°), also examined by Kuhn, Lederer & Deutsch (*27*), occurs along with "vitellolutein" in the red eggs of the sea spider (*Maja*

squinada) and represents a hypophasic ester of astacin. Further there also belong to this group the pigments of some sea crabs (*Palinarus vulgaris*) as well as the river crab (*Potamobuis astacus*) and *Leander seratus* and *Portunus puber* as investigated by Fabre & Lederer (28).

Lyochrome

This designation was given by Ellinger & Koschara (29) in agreement with Kuhn, György & Wagner-Jauregg (30)[2] to a group of pigments that occur in the liver and kidneys of frogs, rats and mice, as well as in cow and human milk. According to von Euler & Hellström (32) they are also to be found in the eggs of *Myxine glutinosa*. The single representatives of the group are named flavines from their origin, or more accurately "lactoflavine" or "ovoflavine." The lyochromes are characterised by their solubility in water and their yellowish-green fluorescence near the neutral point, as well as the reversible reduction to leuco compounds by the action of sodium hyposulphite or hydrogen sulphide in sodium carbonate solution. These leuco compounds suffer dehydrogenation to the coloured form even in the oxygen of the air. Lactoflavine-*a* crystallises from water as red or reddish-yellow spherical masses, while the *b* variety also crystallises from water in colourless plates. Lactoflavine-*b* and -*c* are very similar in composition to uric acid but differ in their remarkable behaviour toward the murexide test. Lactoflavine-*c*, whose composition is given by the formula $C_{19}H_{18}O_{12}N_{14}$, crystallises as reddish-brown plates without a melting-point. It is far less soluble in water than lactoflavine-*a* and -*b*. The heating of lactoflavine-*b* splits it into a purine-like body and lactoflavine-*d* ($C_{16}H_{20}O_6N_4$ or $C_{17}H_{20}O_7N_4$). Lactoflavine-*d* is more easily soluble in water than -*a*, -*b*, or -*c*, and crystallises out into yolk-yellow soft needles or deep red rods prior to decomposition.

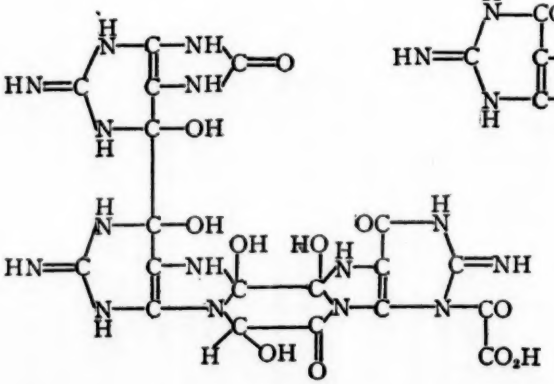
Pterines

Wieland (33) formerly supported the view that the constitution of leucopterine, the white wing pigment of the butterfly (*Pieridae*) was to be represented as that of a dipurine $(C_5H_5O_3N_4)_2$. The recent investigations of Wieland, Metzger, Schöpf & Bülow (34) now

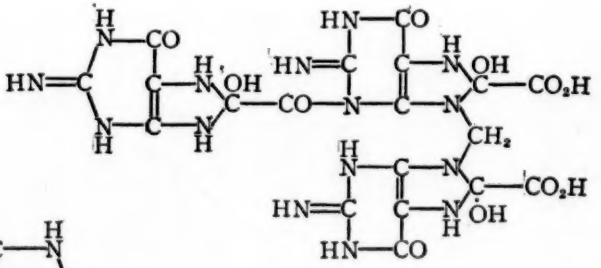
[2] Compare also Warburg & Christian (31).

show, however, that this conception cannot be maintained, since the presence of guanidine was demonstrated in the degradation of the leucopterine.

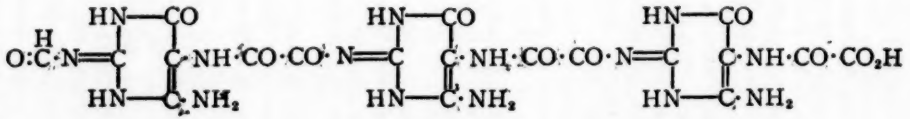
It now seems likely that one is dealing with a complicated purine derivative with the probable composition $C_{19}H_{19}O_{11}N_{15}$. It is a combination of three purine nuclei which, on the one hand, bears an affinity to guanine, due to the guanidine groups, and on the other, to uric acid, due to the oxidation state of carbon atom 8. The linkage of the purine nuclei is very probably to be sought at the carbon atoms 8 and 6, partly in direct coupling or perhaps through the parts of the molecule that must be accounted for on the remaining four carbon atoms. A part of the purine carbon comes from the three molecules of oxalic acid that can be hydrolytically split from leucopterine. The 15 nitrogen atoms belong to the three imino purine nuclei and they all have the oxidation state of ammonia. The following formulae (XVI, XVII, and XVIII) have been proposed for leucopterine. The researches of Schöpf & Becker (35) have shown that the formula of a dihydrodixanthine originally proposed for xanthopterine, the first crystalline pigment isolated by Wieland & Schöpf (36) from the wings of *Gonopteri rhamni,* can now no longer be supported. From the composition of the barium salts, α- and β-, it was concluded that there are six acidic hydrogen atoms in xanthopterine, or more accurately in the β barium salt of the xanthopterine monohydrate from which it is derived. Of these six atoms at least four are to be ascribed to CO-NH- groups. The same workers also succeeded in showing that the yellow pigment of the wasp (*Vespa*), which is largely localized in the horny shell of the abdomen, is identical with xanthopterine.



XVI



XVII



XVIII

Formulae proposed for leucopterine

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ANIMAL PIGMENTS*

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The pigments considered in the first part of this review are the pigments found in vertebrate and invertebrate bloods. The pigments considered in the second part are tissue enzymes. Most of these blood and tissue pigments are concerned either with the transportation of oxygen or with the catalysis of cellular oxidations. The main advance in the recent study of animal pigments has been the demonstration that important tissue enzymes are pigments and that some of these enzymes are closely related to hemoglobin.

Respiratory Pigments of the Blood

Respiratory function of hemoglobin in the blood.—In 1928 the problem of carbon dioxide transport in blood was generally believed to be solved. It was supposed, on the basis of highly accurate experiments, that under physiological conditions carbon dioxide is carried by the blood as dissolved carbon dioxide, carbonic acid, and bicarbonate, and in no other form. It had previously been held by some physiologists (notably by Bohr, 1909) that part of the carbon dioxide combines with hemoglobin to form carbhemoglobin, a compound analogous to oxyhemoglobin. This theory was thought to be disproved, some of the most conclusive evidence being the experiments of Van Slyke and his colleagues (Peters & Van Slyke). Today the whole problem is seen in an entirely different light, and this is due primarily to Henriques, the first of whose papers on this subject appeared in 1928. Henriques introduced a new point of view, made some important new observations, and also showed that the experimental evidence for the accepted doctrine was not as conclusive as had been supposed. The new point of view was to consider the kinetics of carbon dioxide transport, in particular the speed with which carbon dioxide escapes from the blood. The origin of this point of view may be traced to the appearance in 1924 of a paper by Faurholt (1) on the speed of hydration and dehy-

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dration of carbon dioxide ($CO_2 + H_2O \rightleftharpoons H_2CO_3$). The velocities of these reactions were found by Faurholt to be very slow, so slow that Henriques calculated there would not be enough time for carbon dioxide to escape from the blood in the lungs if the speed of dehydration of carbonic acid were the limiting factor. Henriques then measured the rate at which carbon dioxide escapes from blood and found that it is in fact much more rapid than that at which carbon dioxide escapes from a carbonic acid–bicarbonate system. Carbon dioxide was found to escape from hemoglobin solutions with the same speed as from blood. This high velocity, Henriques realized, could be explained by supposing either that a catalyst is present or that part of the carbon dioxide in blood and in hemoglobin solutions is in some form other than carbonic acid and bicarbonate. The evidence he possessed led him to favor the latter interpretation, that part of the carbon dioxide is in the form of carbhemoglobin. Faurholt (2) had found that the rates of formation and decomposition of carbamino compounds are very rapid:

$$RNH_2 + CO_2 \rightleftharpoons RNHCOOH$$

Henriques consequently supposed carbhemoglobin to be a carbamino compound.[1]

The development of the investigation begun by Henriques has proceeded along two distinct lines; it has been found, on the one hand, that there exists in the blood an enzyme catalyzing the hydration and dehydration of carbon dioxide, and on the other hand, convincing evidence for the existence of carbhemoglobin has been presented.

An enzyme has been prepared from blood [independently by Meldrum & Roughton (1), and by Stadie & O'Brien (2)] which catalyzes both the hydration and dehydration of carbon dioxide. The enzyme, called carbonic anhydrase, is present in red blood cells and not in plasma. It has been prepared free of hemoglobin. Since its action is inhibited by cyanide and carbon monoxide, it is probably an iron pigment. In considering the physiological significance of the enzyme it should be noted that the activity of unlaked corpuscles is only 0.01 to 0.05 per cent of the activity of the same amount of laked blood.

Part of the evidence for the existence of carbhemoglobin has come from investigations of equilibrium conditions in systems containing

[1] It is perhaps worth noting that Bohr, Faurholt, and Henriques have all done their work in Copenhagen.

hemoglobin and carbon dioxide. Hemoglobin appears to change the first dissociation constant of carbonic acid, represented as pK'_1 in the Henderson-Hasselbalch equation

$$pH = pK'_1 + \log (CO_2 \text{ combined}) - \log (CO_2 \text{ dissolved})$$

That pK'_1 is diminished by the presence of hemoglobin was first shown by Van Slyke and his collaborators. This effect of hemoglobin can be attributed either to a depression of the activity coefficient of bicarbonate ions, by hemoglobin, or to the combination of carbon dioxide with hemoglobin. Either explanation accounts for the diminution of the value attached to (CO_2 combined) in the above equation. Van Slyke and Stadie and their collaborators [Van Slyke, Hastings, Murray & Sendroy; Stadie & O'Brien (1)] favor the former explanation, whereas Henriques, and Margaria & Green favor the latter. Margaria & Green have made the most exhaustive study of the effect of hemoglobin on pK'_1, and have shown that the assumption that part of the carbon dioxide combines with hemoglobin provides the simplest explanation of the facts. Perhaps the most convincing piece of evidence for the combination of carbon dioxide with hemoglobin is that the combination of oxygen with hemoglobin is affected by carbon dioxide even when the hydrogen-ion concentration and ionic strength are maintained constant. If variations in pK'_1 are attributed to combination of carbon dioxide with hemoglobin, one-third of the carbon dioxide taken up by the blood of the capillaries is in the form of carbhemoglobin.

Further evidence for the existence of carbhemoglobin has come from a continuation [Meldrum & Roughton (2)] of Henriques' investigation of the speed of the carbon dioxide exchange in blood. It was found more convenient to study the rate of uptake of carbon dioxide by carbon-dioxide–free blood than the rate at which carbon dioxide escapes from the blood. Activity of the enzyme carbonic anhydrase was eliminated by completely inhibiting it with cyanide. Blood poisoned with cyanide takes up carbon dioxide in two phases. Of these the rapid one is due in part to physical solution, but in the main to the formation of some non-bicarbonate chemical compound, whereas the second, slow phase is due to the formation of bicarbonate following the reaction $CO_2 + H_2O \rightarrow H_2CO_3$. Experiments on the uptake of carbon dioxide by ammonia, certain amino acids, and peptides give results so very similar to those with cyanide-blood that it is supposed that the same type of carbon dioxide compound is concerned in all

these cases, viz., a carbamino compound. More carbhemoglobin is formed in reduced blood at a given carbon dioxide pressure than in oxygenated blood. All these conclusions are substantially the same as those arrived at previously by Henriques, but the elimination of the enzyme by cyanide greatly clarifies the whole situation. The significance of carbhemoglobin under physiological conditions is however by no means clear.

Barcroft has published the first report of a highly interesting investigation of the respiratory relations between mother and foetus. The demand for oxygen made by the foetus grows quite disproportionately to the supply so that, as pregnancy proceeds, blood leaving the uterus and returning to the mother becomes increasingly dark. Transfer of oxygen from mother to foetus is greatly facilitated by shifts in the oxygen-dissociation curves of their bloods. From about the middle of pregnancy onward, the oxygen-dissociation curve of maternal blood shifts to the right of the normal position, so that oxygen tends to leave the blood more readily than it did previously. This shift may be accounted for by an increase in the hydrogen-ion concentration of the maternal blood. At about the same time, the foetal oxygen-dissociation curve shifts to the left, so that oxygen tends to be absorbed by the blood more readily than before. This shift is due not to a change in alkalinity of the blood but to the altered constitution of foetal hemoglobin. Evidence that foetal hemoglobin differs from normal hemoglobin has also been provided by Haurowitz and by Trought. The latter found that the difference persists in infants for several months after birth. Hemoglobin of the new-born has also been studied crystallographically (Perrier & Jannelli).

Since dwellers at high altitudes, as well as embryos, live in a rarefied-oxygen atmosphere (for a statement of this analogy see Anselmino & Hoffmann), it may perhaps be significant that their hemoglobin differs from normal hemoglobin. The hemoglobins of man and animals at high altitudes have isoelectric points between pH 7.0 and pH 7.2 instead of pH 6.8, the value at sea-level (Rubowitz).

A classification of the respiratory proteins in terms of their sedimentation constants, molecular weights, and isoelectric points has been made by Svedberg after a systematic study of chlorocruorin and of many hemoglobins and hemocyanins. Hemoglobins contained in blood corpuscles, whether of vertebrates or invertebrates, have comparatively low molecular weights, of the order of 68,000. Respiratory proteins dissolved in the blood plasma—chlorocruorin, the hemocyanins, and

many hemoglobins of the invertebrates—have high molecular weights, of the order of one to five million. Svedberg & Eriksson suggest that the respiratory pigment of the invertebrates hitherto known as hemoglobin because of its apparent similarity to vertebrate hemoglobin should be called erythrocruorin instead. This change, it seems to us, is hardly justifiable. The change is proposed because it is supposed that the red pigment of these invertebrates is, in respect to the properties of the protein part of the molecule, more allied to chlorocruorin and to hemocyanin than to the hemoglobin of the vertebrates. The protein properties referred to are the molecular weight and isoelectric point, and yet in the very next paper Svedberg reports that many of the invertebrate hemoglobins have molecular weights of the same order of magnitude as that of the vertebrate hemoglobins. Since the hemoglobins of vertebrates and invertebrates have the same spectroscopic properties and the same reactions with gases it follows, furthermore, that the proteins of these hemoglobins must have certain properties in common. It is only when heme is combined with native globin that it has those properties observed in hemoglobin [Anson & Mirsky (1)]. No other protein now known (except that of chlorocruorin), when combined with heme, causes it to exhibit those properties. The proteins of the various hemoglobins may differ with respect to their molecular weights and isoelectric points but they are all able to unite with heme to form hemoglobins. On the basis of this highly distinctive property, these proteins should all be classified as globins. Whether the proteins of hemocyanins possess this property is not known. It would, indeed, be interesting to know whether the protein of hemocyanin forms a hemoglobin-like compound when added to heme. As for chlorocruorin, it is known [Warburg & Negelein (1); Roche] that the protein of this pigment resembles globin in certain important respects, for, if globin prepared from hemoglobin of the ox is added to the prosthetic group of chlorocruorin, a pigment differing only slightly from chlorocruorin is obtained.

Miscellaneous observations on hemoglobin.—Observations have been made on the respiratory activities of hemoglobins of the following animals: the skate (Dill *et al.*), various marine fishes (Root), the sea-lion (Florkin & Redfield), the echinoid worm—*Urechis caupo* (Redfield *et al.*). In the case of *Urechis caupo,* Baumberger & Michaelis noticed that the blood cells contain free heme in addition to hemoglobin.

It has been supposed (Geiger) that blood contains a mixture of two different hemoglobins, for in cataphoretic experiments on hemo-

globin part of the pigment was observed to migrate toward one pole, while the rest moved toward the other. Dummett & Bowden state that this peculiar behavior of a hemoglobin solution disappears if the solution is passed through a filter candle.

Muscle hemoglobin, about which very little is known, has now been crystallized by Theorell. The molecular weight of the purified protein is 33,000, one-half that of the hemoglobin in the blood of the same animal. A simple relationship exists between the relative affinities of any hemoglobin for oxygen and carbon monoxide on the one hand, and the positions of its oxyhemoglobin- and carbon-monoxide-hemoglobin-absorption bands on the other (Anson, Barcroft, Mirsky & Oinuma). This rule, which holds both for vertebrate and invertebrate hemoglobins, can be extended to muscle hemoglobin (Roche). Muscle hemoglobin has a greater affinity for oxygen than has the hemoglobin in the blood of the same animal (Hill).

The manner in which hydrogen sulfide combines with hemoglobin has been obscure. Treatment of hemoglobin with hydrogen sulfide causes some changes which cannot be reversed, at least it has not been possible to recover unchanged hemoglobin merely on removing hydrogen sulfide. Keilin (1) has observed that this action of hydrogen sulfide on hemoglobin requires the presence of oxygen. Acid methemoglobin on the other hand was found to combine with hydrogen sulfide in a clearly defined and reversible manner, analogous to the way in which it combines with cyanide.

The oxidation-reduction potential of the hemoglobin-methemoglobin system has been measured again by Conant & Pappenheimer. In Conant's earlier experiments, when the attempt was made to titrate hemoglobin electrometrically with ferricyanide and methemoglobin with hydrosulfite, some difficulties were encountered. By simply mixing hemoglobin and methemoglobin in various proportions and measuring the potentials of the mixtures, more reproducible results are obtained.

The difficulties of the direct measurement of potentials have been avoided by Schüler in a most ingenious manner. From measurements of the equilibrium constant of the reaction between carbon-monoxide hemoglobin and ferricyanide and the dissociation constant of carbon-monoxide hemoglobin (both of which constants can be measured manometrically), the potential of the hemoglobin-methemoglobin system can be calculated. An important correction is introduced by recognizing (as other investigators had failed to do) that ferricyanide

reacts with the sulfhydryl groups of globin as well as with the ferrous iron of heme.

Optical properties of hemoglobin and its derivatives have been investigated (Clark & Gibson; Drabkin & Austin; Holden & Hicks; Maltby; Parnas & Sieniawski; Parpart, Amberson & Stewart; Sebesta; Winegarden & Borsook).

Among these contributions will be found many suggestions for the estimation of hemoglobin. For the estimation of traces of hemoglobin by benzidine, see the paper of McFarlane & Hamilton.

Hemoglobin has been used as a well-defined protein for various purposes: for the study of the so-called dissociation tendency of proteins (Sörensen & Sörensen); to demonstrate the influence of the underlying surface on the cataphoretic mobility of adsorbed proteins (Dummett & Bowden); as a substrate for the estimation of pepsin and trypsin [Anson & Mirsky (2)].

Chlorocruorin, hemocyanin, and hemoerythrin.—Considerable progress has been made in the study of these three protein respiratory pigments of the invertebrates. Fox found that the oxygen-dissociation curve of chlorocruorin is sigmoid, like that of hemoglobin. Fox & Roche prepared chlorocruorin in crystalline form.

In their study of the way in which acid diminishes the affinity of *Limulus* hemocyanin for oxygen, Redfield, Mason & Ingalls have supposed that acid affects hemocyanin in the same way as it does hemoglobin. In the case of hemoglobin the effect of acid has been explained on the assumption that the salt and acid forms of hemoglobin have different affinities for oxygen. In the case of hemocyanin, Redfield and his collaborators claim to have separated definitely the "salt" from the "acid" form of hemocyanin, and accordingly believe that they have secured "more concrete evidence for this aspect of the theory of the oxygen-dissociation curve than has heretofore been obtained." The actual observations of Redfield and his colleagues are that hydrochloric acid converts hemocyanin into a colorless compound that cannot combine with oxygen, that is precipitated from acid solution by adding a small amount of sodium chloride (an amount too small to precipitate the blue form of hemocyanin) and that is also precipitated (instead of being reconverted into the soluble blue form of hemocyanin) by neutralizing with alkali. Difficulties are encountered in attempting to interpret these observations on the assumption that the colorless form of hemocyanin is merely a protein salt. Another interpretation, not mentioned by Redfield and his collaborators, is that the colorless

form of hemocyanin produced by hydrochloric acid is denatured hemocyanin. In this way, all the observations described can be readily explained. It may be added that at the hydrogen-ion concentration of many of these experiments, pH 3, hemoglobin, too, would be denatured.

The speeds with which oxygen combines with hemocyanin and dissociates from oxyhemocyanin were measured by Millikan, by means of the same methods that Hartridge & Roughton used for hemoglobin.

By using powerful oxidizing agents, Conant, Chow & Schoenbach were able to oxidize hemocyanin to methemocyanin, a compound analogous to methemoglobin. Unlike methemoglobin, however, methemocyanin combines reversibly with oxygen; the deoxygenation of methemocyanin like that of hemocyanin may be brought about by diminishing the partial pressure of oxygen above the solution.

The prosthetic group of hemocyanin has been isolated in crystalline form. Heretofore there has been little evidence that hemocyanin possesses a prosthetic group that, like heme of hemoglobin, can be split off from the protein part of a molecule. Schmitz has isolated a substance, which he calls hemocuprin, containing nitrogen, 6 per cent of copper, and no sulphur. This substance is probably the same as the one prepared later by a different method and in crystalline form by Laporta. To separate hemocuprin from the protein attached to it, Laporta used the same acid-acetone procedure as had Anson & Mirsky to separate heme from globin. That the hemocuprin of Schmitz and the crystalline pigment of Laporta are the same and that they are the prosthetic group of hemocyanin was shown by Roche & Dubouloz (1). They observed, confirming Dhéré, that hemocyanin contains in the ultra-violet two bands, only one of which can be attributed to the protein part of the molecule. On examination of the ultra-violet absorption spectra of the pigments of Schmitz and Laporta, only one band was found. This was in the same position as that band of reduced hemocyanin which was not due to protein. Conant, Chow & Schoenbach believe that they too have isolated the prosthetic group of hemocyanin. The substance they prepared contains sulphur and about 20 per cent copper, a composition markedly different from the hemocuprin of Schmitz and Laporta.

By use of the acid-acetone procedure Roche & Dubouloz (2) succeeded in crystallizing the iron-containing prosthetic group, hemoferrin, of hemoerythrin. Its absorption spectrum in the ultra-violet resembles that of hemocuprin. At about the same time hemoerythrin itself was prepared in crystalline form by Florkin in the course of a thorough study of this pigment.

Chlorocruorin is certainly very similar to hemoglobin [Fox; Warburg & Negelein (1); Roche]. It is, however, highly conjectural how hemocyanin and hemoerythrin are to be classified with respect both to their proteins and prosthetic groups. Roche suggests that their prosthetic groups have similar structures, but Florkin sees no reason to think so. Now that both the pigments themselves and their prosthetic groups have been crystallized, we may expect that the question of classification will soon be settled.

Vanadiumchromogen.—The respiratory pigment in the blood of ascidians bears scarcely any resemblance to the respiratory proteins which have just been described. Vanadiumchromogen is an organic compound containing 10 per cent of vanadium (Henze). The pigment, insoluble in neutral solutions, is soluble in 3 per cent hydrochloric acid; it is, in fact, dissolved in 3 per cent sulfuric acid within the blood cells. On the other side of the cell membrane is an approximately neutral plasma, and yet the membrane is so delicate that it is disrupted simply by adding distilled water. Vanadiumchromogen does not form a loose combination with oxygen. It is, on the other hand, a reducing agent, instantly reducing ferri-salts, permanganate, chromate, methylene blue, or indigo. The existence of vanadiumchromogen reminds us that, chemically as well as morphologically, life assumes some very bizarre forms.

Animal Pigments as Enzymes

A few enzymes have been isolated and shown to be proteins. Nothing, however, is known about the chemical structures responsible for the catalytic activities of these protein compounds. On the other hand, it has been possible to identify the so-called active groups of a few enzymes because of the fortunate fact that these active groups are pigments. To what sorts of substances these pigments are joined in the intact enzymes which do not consist of the pigments alone is not known.

The yellow enzyme.—One would expect the oxidative enzymes which are inhibited by cyanide to be heavy metal compounds. About the nature of those oxidative enzymes which are not inhibited by cyanide no a priori prediction can be made. Warburg & Christian have now identified one such enzyme. It contains no heavy metal, which accounts for its not being inhibited by cyanide and carbon monoxide. In its reduced form, like leucomethylene blue, it is practically colorless. In its oxidized form it is yellow with a green fluorescence. The active enzyme, which has not yet been prepared in pure form, consists of the

pigment joined to a non-diffusible substance of unknown nature. When the pigment is freed from the non-diffusible component of the enzyme it no longer can catalyze oxidations, just as heme can no longer combine loosely with oxygen when it is freed from globin. The properties of an enzyme are properties of the molecule as a whole, not merely of the so-called active group.

In general, unicellular organisms which are aërobic and have a large cyanide-sensitive respiration have relatively little of the yellow enzyme, while anaërobic unicellular organisms whose oxidation-reduction processes are less sensitive to cyanide are relatively rich in the yellow enzyme. An example of the latter class is anaërobically cultivated lactic-acid bacteria whose oxygen consumption is not affected by cyanide. When these bacteria are exposed to oxygen, one can see the yellow color of the enzyme. When conditions are made anaërobic, the yellow color disappears. Normally the bacteria are not exposed to oxygen at all, so normally the yellow enzyme must be oxidized by some cellular oxidant other than oxygen. Thus the enzyme is probably concerned with those oxygenless oxidation-reduction processes which have been studied by the hydrogen acceptor school.

Under suitable conditions the yellow enzyme can catalyze oxidations *in vitro*. Warburg & Christian have studied in detail the oxidation of hexosemonophosphate.

The universality of the yellow pigments.—Ellinger & Koschara, and Kuhn, György & Wagner-Jauregg simultaneously pointed out that Warburg & Christian's yellow enzyme is one of a group of related pigments which are present in animal tissues generally. Ellinger & Koschara came upon the yellow pigments while studying the green fluorescence of tissues, and Kuhn and his co-workers while studying vitamin B_2, the anti-pellagra vitamin, which apparently is closely related to, if not identical with, the yellow enzyme. During the past few months many papers concerned with this new group of pigments have appeared [Ellinger & Koschara; Euler & Hellström; Kuhn, György & Wagner-Jauregg; Kuhn & Wagner-Jauregg; Stern (3); Stern & Greville; Wagner-Jauregg & Ruska]. Most of these papers are hurried accounts of work in progress. A detailed consideration of the whole subject had best be postponed until the next review. In the next two years the precise chemical nature of the yellow enzyme and of vitamin B_2 will probably be worked out.

Inhibition by cyanide and carbon monoxide.—[See the reviews of Keilin (2), and Hand.] At the time of our previous review the cur-

rent views about the reactions of cyanide and carbon monoxide with the generally occurring cellular iron-porphyrin compounds were briefly these. (*a*) There is an essential respiratory enzyme which is oxidized by molecular oxygen. The spectrum of this enzyme's carbon-monoxide compound can be learned by measurement of the effect of visible light on the carbon-monoxide inhibition of respiration. (*b*) Cytochrome whose oxidation is inhibited by cyanide and carbon monoxide does not itself react with these inhibitors. (*c*) Catalase and peroxidase are heme compounds which are inactive when combined with cyanide.

As regards the measurement by the light method of the spectrum of the respiratory ferment which reacts with oxygen and carbon monoxide, Kubowitz & Haas in an important technical paper have described methods for obtaining intense monochromatic lights of many wave-lengths, and they give the spectrum of the respiratory ferment in great detail. Schmitt & Gasser have studied the effect of light on carbon-monoxide inhibition in nerve.

Warburg & Negelein (2) claim that in the favorable case of acetic-acid bacteria, which have an intense respiration, one can see directly the spectrum of the carbon-monoxide compound of the respiratory ferment. Keilin (3) disputes this claim that the pigment seen is the respiratory enzyme which reacts with oxygen. He states that in certain cases the components of cytochrome can themselves combine with carbon monoxide and that carbon monoxide does not change the visible spectrum of azotobacter which respires more intensely even than acetic-acid bacteria.

Warburg & Negelein (2) have also observed a band in the red which appears on the addition of cyanide and whose appearance is accompanied by inhibition of respiration. They believe this band to belong to the cyanide compound of the ferri form of a heme which is separate both from cytochrome and from the heme ferment which reacts with oxygen. The evidence given for this view is not convincing. Keilin (3) believes that the band in the red belongs not to a cyanide compound at all but to the ferro form of a component of cytochrome.

It is clear that the heme pigments which can be seen in cells are very variable (Fink & Berwald) and that they deserve more precise study. In this study it is to be hoped that the relation of the pigments to respiration rather than to some arbitrary conception of cytochrome will be emphasized.

Stern (1), who has written several papers on catalase, describes experiments on the effect of cyanide and carbon monoxide. Haurowitz

(2) has studied recrystallized hemoglobin as a catalase. Elliott & Sutter have shown that the inactivation which Kuhn observed on shaking peroxidase with carbon monoxide was due to the shaking, and not to the carbon monoxide as had been stated.

In studying the reactions of carbon monoxide with heme pigments it is not sufficient to measure merely the disappearance of carbon monoxide. Fenn & Cobb found that muscle contains an enzyme system which can catalyze the oxidation of carbon monoxide. Negelein (1) found that certain heme compounds can likewise catalyze the oxidation of carbon monoxide.

Inhibition of butyric-acid fermentation by cyanide and carbon monoxide.—Glycolysis can be inhibited by cyanide, but it takes a much higher concentration of cyanide than is needed for the inhibition of respiration. When it was discovered that carbon monoxide can inhibit respiration, the attempt was made to inhibit glycolysis by the same reagent. No inhibition was observed even when cells were exposed to carbon monoxide at many atmospheres pressure. Recently Kempner discovered that cyanide inhibits the formation of acid by butyric-acid bacteria even in the low concentration in which cyanide usually inhibits respiration. He accordingly tried the effect of carbon monoxide and found that the acid formation is likewise inhibited by carbon monoxide. Later Kempner & Kubowitz found that this carbon-monoxide inhibition can be decreased by visible light, although a very much stronger light is needed than for the abolition of the carbon-monoxide inhibitions of respiration. It is thus proven that some reaction involved in butyric-acid fermentation (not necessarily the hydrolysis of carbohydrate itself) is catalyzed by an iron-containing ferment, for iron is the only heavy metal whose carbon monoxide compounds are dissociated by visible light. Since there are enzymes of the same general type involved in both respiration and butyric-acid fermentation, there must also be mechanisms of the same type involved in the two processes. Whether this same type of mechanism is also involved in ordinary glycolysis remains to be seen. Lipmann has recently presented evidence that an oxidation-reduction process controls glycolysis. It is clear, in any case, that Kempner's notable discovery has opened a new chapter in the study of animal pigments.

Hemes and their nitrogenous substances.—The peculiar properties of the heme enzymes are partly due to their iron-porphyrin or heme nuclei and partly to the nitrogenous substances to which the hemes are joined. In general two types of hemes are known to be of general

occurrence in nature, one similar to the heme of hemoglobin, the other similar to the heme of chlorocruorin [Warburg & Negelein (1)] and of the respiratory ferment which reacts with oxygen and carbon monoxide. Anson & Mirsky (1) showed that when yeast and bee's muscle (which is hemoglobin-free) are extracted with pyridine, two different hemochromogens are obtained, one similar to the hemochromogen formed by the addition of pyridine to the heme of hemoglobin, the other similar to that formed by the addition of pyridine to the heme of chlorocruorin. Since it was not certain that pyridine completely displaces the unknown nitrogenous substances of yeast and bee's muscle, the suggestion was made that, before the addition of pyridine, the hemes be first separated from their nitrogenous substances by acid acetone. Negelein (2) has now isolated from an acid acetone extract of heart muscle a heme similar to the heme of chlorocruorin, and he has prepared from it a crystalline pyridine hemochromogen.

According to Hill & Keilin, one hydrogen equivalent is required for the oxidation or reduction of component *c* of cytochrome just as is the case with hemoglobin itself or its heme.

There has been no direct chemical information about the nitrogenous substances to which the heme pigments of general occurrence are joined because none of these compounds of heme and a nitrogenous substance has as yet been isolated in pure form. Attempts have been made, however, to determine the molecular weights of two of these compounds by the diffusion method of Northrop & Anson, which can be applied even to impure substances in unknown absolute concentrations. The results are as yet uncertain. In the case of catalase, Zeile states that catalase is inactivated by the dilution incident to diffusion. Stern (2) states that under the conditions chosen by him there is no such inactivation and that catalase has about the same molecular weight as hemoglobin. Zeile & Reuter have measured the rate of diffusion of component *c* of cytochrome, which they used in a considerably but not completely purified form. They feel uncertain about the interpretation of their measurements because of the possibilities of aggregation and of combination with all sorts of extraneous substances. These possibilities could probably be eliminated by carrying out diffusion experiments in different salt solutions and by testing the homogeneity in size of the component-*c* molecules.

The respiration of red blood cells.—(See the review of Rosenthal.) Barron & Harrop discovered that red blood cells, which normally do not respire, consume oxygen when methylene blue is added. Warburg

and his co-workers showed that methylene blue oxidizes hemoglobin to methemoglobin and is then reoxidized by oxygen. The methemoglobin formed oxidizes some constituent of the red blood cell and is thus once more reduced. In balance, then, the cell constituent is oxidized by oxygen, methylene blue, and hemoglobin acting as catalysts. Reagents other than methylene blue which form methemoglobin also cause oxidation. When the formation of methemoglobin is inhibited by carbon monoxide, the oxidation caused by methemoglobin is, of course, prevented. This work, which was described in our previous review, has now been presented in greater detail (Warburg, Kubowitz & Christian), and it has also been shown that methylene blue in higher concentrations can catalyze oxidation in the blood cell directly without the formation of methemoglobin. Under these conditions carbon monoxide has no inhibiting effect. Wendel has likewise proved this direct action of methylene blue by showing the absence of inhibition by cyanide, which inhibits completely the oxidations by methemoglobin. Friedheim has shown that there are naturally occurring animal pigments which can act like methylene blue in causing red blood cells to respire.

Methylene blue and cyanide poisoning.—Sahlin and later others have observed that methylene blue is an antidote to cyanide poisoning in those happy cases in which it can be given promptly enough. Methylene blue acts by forming methemoglobin, which has a high affinity for cyanide. When methemoglobin is present, cyanide combines with this pigment rather than with enzymes which are essential to life. Any reagent which forms methemoglobin has the same effect as methylene blue, and some of these reagents are much more effective than methylene blue. References to the numerous papers on this subject can be found in the very recent note of Chen, Rose & Clowes.

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BIOCHEMICAL AND NUTRITIONAL STUDIES IN THE FIELD OF DENTISTRY*

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Bödecker (12) reviewed the early work on the chemical composition of teeth. Kaushansky (67) recently reported an average of 28.46 per cent of calcium in the whole normal tooth, compared with 31.55 per cent in the crown and 25.37 per cent in the root. Phosphorus was found to be distributed in the whole tooth, crown, and root in average amounts of 14.32, 15.65, and 13.0 per cent respectively, while the concentration of magnesium was 0.60 per cent in the whole tooth, 0.50 per cent in the crown, and 0.71 per cent in the root, confirming the early work of Gassmann (40). Kaushansky's analyses of carious teeth showed more phosphorus and magnesium and slightly less calcium in the crowns with little change in the composition of the root, confirming the earlier work of Howe (51) and Ulrich (116). In pyorrhoea, however, Kaushansky found a marked increase in the magnesium content, but almost no change in the calcium and phosphorus content of the root. The composition of the crown, in cases of pyorrhoea, was intermediate between that of normal and carious teeth.

Thewlis (112) used X-ray methods of studying teeth. He stated that apatite is the only crystalline constituent of enamel and dentin present in appreciable amount. Comparative studies of the density of enamel and dentin of human and dog teeth indicated about 0.5 per cent of organic matter in enamel and 40.0 per cent in dentin.

Relation of Food and Metabolism to Tooth Formation

Age periods for calcification of human teeth.—Hess, Lewis & Roman's analyses of deciduous teeth of still-born infants (47) showed that only 20 per cent of the calcium ultimately to be found in the erupted teeth had been laid down in the central and lateral incisors, 21 per cent in the first molars, 11 per cent in the second molars, and 7.5 per cent in the canines. Only 0.1 per cent of the calcium later to be found in the first permanent molar was present in this tooth at birth. These observations and those of Kugelmass & King (75) point to the futility of efforts to improve the permanent teeth of children through improving maternal nutrition during the gestation period.

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Logan & Kronfeld (77) gave the following age periods for beginning calcification of the permanent teeth: six-year molars, 6 months; cuspids and lower lateral incisors, 3 to 6 months; central incisors, 1 year; upper lateral incisors, 1 to 15 months; bicuspids and second molars, 1.5 to 2.5 years; and third molars, 7 to 9 years. Meany (88) has reported studies indicating that the periods of most active calcification of the permanent teeth are: six-year molars, central and lateral incisors, and cuspids, the first three years; bicuspids and second molars, 3 to 6 years; third molars, 9 years on.

Observations on experimental animals.—In 1928 Mellanby (89) reviewed the work that she and others had done, demonstrating the close relation between diet and the formation of normal teeth. Since this publication many studies have been conducted on experimental animals, using as criteria the effects produced by different diets on the incisor teeth of rats and on the teeth of guinea pigs, since, in these species of animals, they represent teeth that are being constantly renewed.

(*a*) *Rôle of calcium, phosphorus, and vitamin D.*—Karshan & Rosebury (62, 65, 100) and Templin & Steenbock (111) fed young rats low-calcium rickets-producing diets. The percentages of ash, calcium, and phosphorus of the incisors were lower than when either calcium or vitamin D was added to the diet. The corresponding percentage reductions found in the tibiae were considerably greater, however, indicating that growing teeth are less sensitive to mineral deficiencies in the diet than is bone. Becks & Ryder (4) in the examination of incisors of rats that had been maintained on a rickets-producing diet for varying periods of time, demonstrated progressive decrease in deposition of calcium salts in dentin, continued formation of dentinoid matrix, and final atrophy of the odontoblasts with cessation of calcification and of the formation of cementing substance. They state that the changes observed in dentin were analogous to those produced in long bones under similar conditions. Similar observations have been reported by Karshan & Rosebury (64, 65) and Blackberg & Berke (11).

Rosebury & Karshan (99, 100) found that the enamel of rat molars and the fully formed enamel of the incisors were, however, unaffected by diets low in calcium and vitamin D. Blackberg & Berke (10) confirmed the findings of Mellanby (89) as to the relative ease of production of hypoplastic enamel in dog teeth by interference with the intake of calcium, phosphorus, or vitamin D.

(*b*) *Rôle of vitamins A and C.*—Recently Day (25) found that continued ingestion of scorbutic diets by pregnant guinea pigs resulted in more noticeable effects on the dentin of maternal teeth than on fetal teeth.

Smith (107) reported changes in composition of rat incisors brought about by diets deficient in vitamin A. She found a decrease in dry weight in comparison with that found in the incisors of control animals. The percentages of ash and of phosphorus were slightly reduced, while the calcium was increased from 37.9 per cent of dry weight in the control animals to 42.2 per cent in the incisors of the experimental group.

Observations on human beings.—The observations of Eliot and colleagues (30), of Hess (cf. 30), and of Mackay & Rose (85) indicate a higher incidence of hypoplastic enamel in children having a history of early rickets than is found in similar non-rachitic groups. Jones, Larsen & Pritchard (59), on the contrary, claim that enamel formation is affected by different metabolic conditions than are bone and dentin. They believe that defective dentin formation is associated with abnormalities in bone formation but that hypoplasia of enamel is related to insufficient base-forming elements in the diet. They have produced no analytical evidence in support of this latter claim.

That vitamin-A deficiency may be a cause of some of the cases of dental hypoplasia in man is claimed by Boyle (18) as a result of his examination of the six-year molars in a three-and-one-half-months-old infant who died, having a history of vitamin-A deficiency.

Mottled teeth.—The condition known as mottled teeth, which was originally described in this country by Black & McKay (9) and by Gasparaini & Piergili (39) in Italy, has recently come into considerable prominence owing to the discovery of its apparent relation to the fluorine content of drinking water.

In a survey of 100 public and 55 private water supplies in Arizona, Smith & Smith (109) found that, in those places where there was a high incidence of marked mottling of enamel, the fluorine content of the water ranged from 5.0 to 15.0 or more parts per million. Where this dental defect was mild, but the incidence high, fluorine was present in the water in amounts varying between 2.7 and 4.0 p.p.m., while occasional occurrence of mottled teeth was associated with the use of water containing 1.5 to 2.7 p.p.m. of this element. No mottling of enamel was found when the water used contained less than 1.3 p.p.m. of fluorine. Somewhat similar findings were reported earlier by

Churchill (23), and McClure came to the same conclusion in his careful review of the subject (80).

Smith, Lantz & Smith (108) found that the sensitivity of the enamel of rat incisors to fluorine present in drinking water was much less when the diet of the animals was adequate than when it was deficient. This may account for the occasional occurrence of mottled teeth in children where the water supply contains less than 2.7 p.p.m. of fluorine.

Recently McKay (84) made a survey of the teeth of the children living in Oakley, Idaho, where the water supply had been changed seven and a half years previous from one containing 6.0 p.p.m. of fluorine to one containing only 0.5 p.p.m. of this element. McKay's survey revealed not a single instance of mottled teeth among the children who had been born during this time, compared with a 100 per cent incidence of mottled teeth found in 1925.

A survey of the distribution and approximate severity of mottling of teeth in different parts of the United States has recently been reported by Dean (26) of the United States Public Health Service.

The histological studies of Williams (117) and of others show clearly that the enamel of mottled teeth is hypoplastic and contains rods and inter-cement substances which are imperfectly calcified.

The similarity of mottled teeth to those produced by parathyroidectomy, as reported by Erdheim (34), led Hauck, Steenbock & Parsons (45) to examine the parathyroid glands of animals fed toxic doses of sodium fluoride. No changes were observed in the glands and the conclusion was drawn that fluorine does not affect calcium metabolism through disturbance of this gland. The manner in which fluorine interferes with proper calcification of enamel during the developmental period has not been definitely established.

McCollum, Simmonds, Becker & Bunting (83) showed that the inclusion of sodium fluoride in the diet of rats to the extent of 500 p.p.m. produced soft, friable teeth. Color changes were noted in the teeth of rats fed diets containing 160 p.p.m. of this salt. Sebrell and colleagues (104) and Sharpless & McCollum (105) stated that small amounts of fluorine are not necessary to the formation of good teeth and that a low fluorine content of bone is not disadvantageous. McClure & Mitchell (81) noted an interference with the deposition of calcium in bone when large doses of fluorine were fed. The minimum amount of fluorine needed to produce enamel defects has already been discussed.

The evidence submitted seems to offer conclusive proof that variable quantities of fluorine in the drinking water may affect the development of the teeth of infants and young children and that this effect is enhanced by nutritional deficiencies. Normal diets may minimize these dental stigmata at fluorine levels on a border line. When the fluorine content of the water is high enough, mottling develops regardless of the quality of the diet.

Complete control at the present time lies in changing the water supply to one which does not contain large amounts of fluorine. There is no method known now by which large quantities of water can be treated so as to throw the fluoride out of solution.

Relation of Nutrition and Metabolic Processes to the Integrity of the Erupted Tooth

All evidence points to the existence of a close relationship between nutritional and metabolic factors and the production of normal teeth. What happens to a non-growing tooth after eruption is another story. Perfection of tooth structure is not sufficient protection against dental caries or enamel erosion. Dental hypoplasia should be carefully differentiated from these two tooth diseases.

Dietary factors.—(*a*) *General balance of the diet:* Hanke and colleagues (42, 43) and Boyd *et al.* (15, 16, 17) regard the erupted tooth as continuously subject to the same nutritional needs and metabolic changes as are other body tissues. They believe that any diet modification that tends to supply something in which the diet had previously been deficient favors continued integrity of the teeth. That such generalizations are not justified by evidence may be seen in recent publications cited at the end of this article (49, 70, 71, 72, 101, 106) in which reports are made of the production of caries in rat molars and in children, while ingesting diets that were apparently adequate nutritionally.

(*b*) *Calcium, phosphorus, and vitamin D.*—Mellanby (89, 90) and others (2, 15, 16, and 102) regard erupted teeth as subject to the same dietary needs and metabolic conditions for normal maintenance throughout life as is bone. Mellanby's observations on different groups of children have been of too short duration to be of conclusive value. McBeath (79), working with a group of institution children for two years, concluded that vitamin D was the most important single dietary factor in tooth protection. Mellanby & Pattison's conclusion (90) that cereals, especially oatmeal, contain an anti-calcifying substance and,

when eaten in considerable amount, increase the need for vitamin D for tooth protection, is not substantiated by the observations of Hewat (48) in New Zealand or by the studies of Koehne, Bunting, and colleagues (70) of orphanage children. This latter group of children had been on a type of diet for a period of years, in which 40 to 50 per cent of the caloric intake came from cereals, yet caries-incidence in this group was remarkably low.

That increasing the mineral or vitamin-D constituent of the diet will not protect rats against tooth destruction produced by variations in particle size of the cereal component of the ration is seen in reports by Hoppert, Webber & Canniff (49) and others (69, 71, 101, and 106). In these studies the ingestion of diets containing certain cereals ground to powder fineness produced no tooth lesions in rat molars, while similar diets containing the same cereal ground to the consistency of fine sand produced caries-like lesions in the molars of all of the animals in eight weeks time.

That destruction of rat molars is not specifically associated with dietary conditions known to be unfavorable for normal calcium and phosphorus metabolism is indicated by the failure to produce caries-like lesions in the molar teeth of rats by feeding "cornless" rickets-producing diets (28, 76, 98, and 99). Evidence has been submitted that human susceptibility to decay of the teeth may prevail when the food intake is adequate and calcium and phosphorus metabolism is normal (72, 55). Also there is evidence that caries-incidence in childhood may be low under dietary conditions not explainable on the basis of intake of calcium, phosphorus, or vitamin D (70).

Arnim and colleagues (3) reported poorly calcified dentin in the molar teeth of rats on diets lacking in calcium and containing low amounts of phosphorus and other minerals. In many instances this dentin had been exposed in the cusps by the wearing down of the enamel layer and had undergone decay. Brooke & Smith (20) reported significant losses in body calcium in rats on similar diets.

(*c*) *Vitamin C.*—Rosebury & Karshan (99) found no relation between the presence or absence of vitamin C in the diet and caries-susceptibility of the rat. This observation has been confirmed by McBeath (78) in studies of institution children. Hanke and colleagues, however (42, 43), believe that orange and lemon juice, if taken daily by children in amounts represented by at least one pint of orange juice, contain something specific in the prevention of dental caries, which may or may not be vitamin C. Results just as satisfactory

as those of Hanke and colleagues in arresting the activity of caries have been obtained over a period of years in an orphanage (70), where the intake of vitamin C was considerably below the recommendations of Hanke or of Howe (51, 52). Therefore, both practically and scientifically, there is still room for skepticism as to the necessity for the daily use of such large amounts of citrus fruit, in attempts to reduce caries-susceptibility.

In these same studies Hanke and colleagues reported complete disappearance of gingivitis with the treatment instituted. Inasmuch as deficiency of vitamin C is associated with increased tendency to gum infection, the above result is of considerable interest.

Composition of blood.—The composition of blood in respect to the calcium and phosphorus content and the alkaline reserve has been regarded by various workers as related to the integrity of the erupted tooth. Such a relationship may be direct, in so far as it determines the formation of secondary dentin (89), or indirect, through determining the concentration of these constituents in the dental lymph (if such a fluid exists), or in saliva.

That significant differences do not exist between the total serum calcium and phosphorus of persons free of caries and of those in whom the disease is active, is indicated by the following reports: 19, 42, 43, 53, 60, 63, and 72. Hanke and colleagues have also found no such differences in determinations of ultra-filtrable calcium and phosphorus of blood serum of children. Mull & Kinney (91) made 4,760 determinations of total serum calcium and phosphorus in 898 women from the twelfth week of pregnancy to the seventh week *post partum*. The range of values was the same for those who developed cavities during the period of observation as for those in whom the disease was inactive.

Benjamin & Hess (7) found that it was a reduction in the proportion of the ultra-filtrable calcium and phosphorus that could be adsorbed by dry barium sulphate, which was the striking characteristic of rachitic blood serum. Benjamin (6) believes that normal calcification of bone is brought about by the absorption by the bone-marrow cells of this ultra-filtrable calcium-phosphorus complex, and that other forms of ionic or ultra-filtrable calcium and phosphorus are not specifically concerned with such processes. If calcification and decalcification of dentin or secondary dentin are intimately related to similar processes in bone, and are directly concerned with the integrity of erupted teeth, further evidence will probably have to be sought in

similar studies of the blood serum of persons whose teeth contain no cavities and of others whose teeth are decaying rapidly.

Neither Hoppert, Webber & Canniff (49) nor others (71) could find any variation from normal in the total serum calcium and phosphorus present in the serum of rats with sound teeth in contrast with that of rats whose teeth were disintegrating rapidly. The diets in all instances were satisfactory for normal growth and well-being. Agnew, Agnew & Tisdall (2) reported low total serum phosphorus to be characteristic of their rats that were developing caries. Their data, however, for animals on diets containing corn, show values ranging from 2.2 to 4.1 mg. of phosphorus per 100 cc. of serum, with almost as wide a range in those groups with a high incidence of caries as in those in which the incidence was low.

Karshan, Krasnow & Krejci (63) and Hubbell (53) were unable to find any correlation between the pH or the carbon dioxide capacity of the blood serum and caries-susceptibility. Jones, Larsen & Pritchard (59) relate the dissolution of enamel so frequently seen in Hawaiian children and which they call odontoclasia, to systemic acidosis, but have presented no data on alkaline reserve values for blood serum in support of their belief.

Metabolic disturbances.—It is a well-known fact that teeth are peculiarly susceptible to decay during puberty. In view of the reports to which reference has already been made, in which maintenance on "cornless" rickets-producing diets failed to produce cavities in the molar teeth of rats, and in which low incidence of caries was observed in children on diets below accepted standards nutritionally, one may well question whether such increased susceptibility during puberty can be explained as due to nutritional inadequacies or to abnormalities in mineral metabolism during this critical period. That it is not specifically related to rate of growth as judged by accepted standards is indicated by the observations of Brodsky (19) and Koehne, Bunting & colleagues (70).

There is a general impression that susceptibility of adults to caries is increased as a result of the strain of pregnancy and lactation. The observations of Mull & Kinney (91) on 358 women throughout pregnancy showed only 15 per cent developing cavities. Balance studies on women during pregnancy have been conducted by Coons & Blunt (24), and others (29 and 86) and during lactation by Macy and colleagues (56 and 86). Losses in mineral matter were noted especially during periods of heaviest milk production, but they were small in propor-

tion to reserve body stores and were reduced by the taking of cod-liver oil and yeast.

Attention is called to the observations of Elliott (31) on a case of renal rickets and of Thoma (113) on a case of hyperparathyroidism. In both instances there was extreme decalcification of the bones without any evidence of caries. Reference has already been made to the fact that even the comparatively labile dentin of teeth is much less susceptible to decalcification processes than is bone.

If pregnancy and lactation are associated with a higher-than-normal incidence of caries, may not the correct explanation have to be sought outside the field of mineral metabolism?

There seems also to be a general impression that persons suffering from uncontrolled diabetes have an increased susceptibility to tooth decay. Biochemical evidence to explain such increased susceptibility, if it exists, is lacking. Boyd, Drain & Nelson (17) have reported success in arresting this susceptibility in patients under dietary treatment for diabetes.

Relation of Biochemical Environmental Factors to the Integrity of the Erupted Tooth

Factors influencing the solubility of enamel and dentin.—One of the prevailing theories today concerning the cause of dental caries centers around the growth on the tooth of colonies of micro-organisms capable of producing acid by the fermentation of carbohydrate and capable of living in acid represented by a pH as low as 3.2. *Bacillus acidophilus* is believed to be the specific organism involved. Evidence for and against this thesis will be found in publications listed at the end of this paper as 32, 41, 57, 58, 70, 71, 72, 76, 96, 114, and 115. In this review we shall be concerned chiefly with problems of a biochemical nature affecting the exterior of the tooth.

(*a*) *Tooth structure.*—Bödecker (13, 14) believes, as a result of dye diffusion tests, that there is a circulation of dental lymph throughout the tooth. Beust (8) regards such evidence as unconvincing. Karshan (61) reported that the pH of the enamel of freshly extracted teeth lies between 7.3 and 7.6, that of dentin between 7.8 and 8.0. Bödecker regards the fact that these values exceed those of plasma as additional evidence for the existence of a mechanism within the tooth for concentrating the acid-neutralizing power of the blood for the protection of the tooth against acids formed on the exterior of the tooth.

Pickerill (95) and others (22 and 117) have observed variations in surface permeability of teeth. They found that fully formed unerupted teeth and those containing enamel defects have a much higher degree of surface permeability than do normal erupted teeth. A convincing explanation of the mechanism by which the permeability of sound teeth is increased after eruption has not yet been offered.

(*b*) *Solubility of enamel and dentin.*—There are various ways of studying this problem. Within the past few years results of three methods of attack have been reported.

Enright, Friesell & Trescher (32) coated sound teeth with asphaltum, leaving only a small area of enamel exposed to various buffer solutions, with and without Ca and PO_4 ions, and representing a wide range of pH values. The criteria used were the appearances of the teeth after staining and examining sections microscopically. Benedict & Kanthak (5) and Forbes (36) determined the percentages of calcium and phosphorus dissolved from weighed portions of dry powdered enamel and dentin in similar series of buffer solutions. All three groups of investigators have concluded that the presence of Ca and PO_4 ions reduces the solubility of enamel and that it is the calcium and phosphorus content of saliva that prevents solution of enamel in that medium at pH values above 5.0. Karshan and colleagues (61, 66) compared the mineral and protein content of water extracts of dry powdered enamel and dentin.

Dobbs (27) immersed freshly extracted teeth in varying concentrations of different acids for different periods of time. He found that the rate of solution depended in part on the acid used and its concentration. However, the completeness of solution depended upon whether or not a portion of the tooth was protected by an impermeable plaque.

(*c*) *Composition and permeability of the plaque.*—Dobbs (27) found that plaques removed from human teeth gave positive color reactions for protein and negative tests for starch. From determinations of glucose after hydrolysis, he estimated that they contained between 27.6 and 52.2 per cent of mucin. The ash content varied from 2.7 to 6.4 per cent and the fat content averaged 1.2 per cent. Enright, Friesell & Trescher (32) express frank skepticism as to the validity of statements assigning to the plaque any degree of uniformity in composition.

Dobbs incubated polished teeth in saliva and bread crumbs at body temperature for many hours, thereby producing plaques resembling

those found on teeth. He also prepared artificial plaques from dried mucin. Nichols, Hatton & Doherty (93) made artificial plaques on clean glass slides by using 6.5 per cent solutions of gastric or salivary mucin in very dilute sodium bicarbonate solution. They tested the relative degrees of solubility of these films in various reagents and found that both had similar properties. Hawkins (46) has asserted that glutin of wheat is the chief constituent of plaques but has given no data in support of his belief. He recommends rigid restriction in cereal intake to prevent plaque formation. Dobbs tested the solubility of plaques in thirteen different reagents possible for use as mouth washes. All were ineffective in removing the films.

Dobbs (27) constructed a diffusion apparatus, using plaques that had been removed from human teeth for his diffusion membrane. Alkaline solutions diffused very slowly but lactic acid dialyzed more rapidly. The rate of diffusion of glucose was one milligram per square millimeter of plaque surface per day. That more studies of this nature will be made soon is indicated by the recent description by Friesell & Enright (37) of a diffusion cell for use with plaques from human teeth.

Chemical composition of saliva.—Marshall (87) gave an excellent review of work reported previous to 1924. There has been considerable interest manifested in such studies during the past few years. Krasnow (73) has demonstrated how necessary it is, if comparable results are to be obtained, that samples be collected at uniform times of day and under uniform conditions of stimulation.

That no relation exists between the calcium and phosphorus content of saliva and the integrity of the erupted tooth is indicated by the work of Youngburg (118) and of Entin & Geikin (33) in studies of the phosphorus content of saliva, by the calcium studies of Horton, Marrack & Price (50), and by the work of Karshan, Krasnow, and colleagues (50, 63, 73) who studied both the calcium and phosphorus content of saliva of persons showing varying degrees of caries-susceptibility.

A knowledge of the acid-neutralizing power of saliva is of interest through the possibility of saliva being able to neutralize acids formed in local areas on the surface of teeth. Also it may bear a relation to enamel-erosion processes. Then the question of the relation between this property of saliva, the alkaline reserve of the blood, and the acid-base value of the diet is of very general interest. Efforts to measure the acid-neutralizing power of saliva have been made along three lines

—pH determinations, and determinations of titratable alkalinity and of carbon-dioxide capacity.

Starr (110) in 1922 reported intensive studies of the pH of human mixed saliva in which he demonstrated many factors that might modify this value appreciably. Hanke & Marberg (42, 43) developed a colorimetric method for estimating the pH of various parts of the mouth. This method was used in studies conducted on 323 children living under uniform conditions. The average values obtained for children representing different degrees of caries-activity were remarkably constant, varying from 6.1 to 6.5. Krasnow (73) and others (19, 53, 63) reported finding no significant differences in pH or titratable alkalinity in the saliva of caries-free individuals compared with that from persons in whom the disease was active. Forbes (35) reported finding a reciprocal relation in individuals, but not from person to person, between salivary pH and the phosphorus and carbon-dioxide capacity of saliva, but does not relate such variations to caries-susceptibility.

Because the carbon-dioxide capacity of blood serum is considered a better single index of its ability to absorb acids than is its pH, some investigators have attempted to evaluate the acid-neutralizing power of saliva in a similar manner. Forbes's work has already been mentioned.

Probably the most extensive studies in the composition of saliva as related to caries-susceptibility have been made by Hubbell. In Table I will be found a condensed statement of the results of studies of the calcium, phosphorus, and carbon-dioxide capacity of the saliva of four groups of children between 6 and 16 years of age. One group (54) consisted of 75 rural public school children, another of 32 city public school children (53). A third group was composed of 12 children selected from a large number living in an orphanage under uniform conditions (70). A fourth group of 20 children also lived under uniform conditions but were on a quantitative diet régime (72). The first two groups of children were under observation for a year and a half, during which at least ten determinations were made for each child. The third group was also under observation for a year and a half, but, because of distance, only two determinations of carbon-dioxide capacity were made for each child. The children in the fourth group were under observation for five to eighteen months, salivary analyses being made at monthly intervals. Paraffin-stimulated saliva was used in all tests and for each group a uniform collection time was observed.

The results provide further confirmation of the findings previously

quoted. While the carbon-dioxide capacity for the susceptible group was definitely lower than for the other two groups, the range for individuals was about the same in all groups, whether classified on the basis of caries-susceptibility or in the original three groups studied. Wide variations in calcium, phosphorus, and carbon-dioxide capacity were found from person to person among the 20 children on controlled quantitative diets. These differences could not be explained by the individual intakes of calcium or phosphorus or by the acid-base value of the food eaten by the individual children. The greatest differences

TABLE I

CARBON-DIOXIDE CAPACITY OF SALIVA AND ITS CONTENT OF CALCIUM AND PHOSPHORUS FOR FOUR GROUPS OF CHILDREN

	Caries-free	Caries-inactive	Caries-active
	(mg. per 100 cc. saliva)		
Calcium,* average	5.4	5.3	5.3
Range	2.8 to 7.6	2.2 to 8.0	2.6 to 7.6
Number of children	30	42	55
Phosphorus,* average	13.8	12.8	13.3
Range	9.6 to 17.8	9.4 to 16.8	9.1 to 16.9
Number of children	30	42	55
	(cc. CO_2 per 100 cc. saliva)		
CO_2 capacity,† average	30.5	27.8	23.4
Range	20.1 to 45.9	15.4 to 48.5	8.3 to 50.9
Number of children	17	15	32

* Salivary calcium and phosphorus were not determined for any of the children in the orphanage group (70).

† Salivary carbon-dioxide capacity was not determined for any of the children in the rural-school group (54).

between the carbon-dioxide capacity of the saliva of children free of caries as contrasted with those in whom the disease was active was found among the 32 city school children, the least differences in the orphanage group.

A general conclusion, therefore, seems warranted, that there is no correlation between the composition of saliva as regards its calcium and phosphorus content or its acid-neutralizing power, and either caries-activity or diet.

In regard to the enzyme activity of saliva, attention is called to the review presented by Marshall (87) in 1924 and to that of Adams & Myers (1). These latter workers have reported studies (1, 92) indicating a possible relationship between the starch-digesting power of water dilutions of saliva, and the chloride and phosphorus content, in connection with caries-inactivity or -activity. Hubbell (53), in studies of a larger group of children under observation for a year and a half, was unable to confirm the existence of such a correlation.

Krasnow & Oblatt (74) reported studies of the protein content of saliva, based on tyrosine determinations. More work should be done on salivary protein, in view of the probability that mucin is an important constituent of plaques. Work needs to be done, also, on methods of measuring the viscosity of saliva and of studying the relation of this factor to the health of oral tissues. Standardized methods for measuring volume flow need to be developed, in order that comparable data may be collected concerning the extent to which this factor is of importance in protecting teeth and oral tissues.

Carbohydrate content of the diet.—This dietary factor enters into the problem through the influence it may have either on the intake of an adequate diet or on enabling aciduric organisms to thrive in the oral cavity and intestinal tract. The relation of the general balance of the diet to dental problems has already been discussed. Attention has been called to the work of Hoppert, Webber & Canniff and others concerning the relation of the particle size of the cereal component of the diet to production of caries in rat molars. A discussion of the relation of the presence of aciduric organisms in the mouth to dental caries is outside the province of this review. Readers are referred to publications listed at the end of this paper numbered 70, 71, and 72 for a discussion of the general relation of the carbohydrate content of food to dental caries.

In conclusion, for the benefit of those who are particularly interested in the etiology of dental caries, recent reviews of the various theories are suggested numbered 21, 32, 38, 42, 44, 52, 68, 71, 82, 94, 97, and 103.

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THE TERPENES, SAPONINES, AND CLOSELY RELATED COMPOUNDS*

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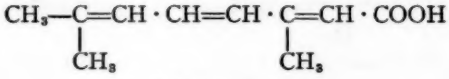
The limits of the field to be reviewed were given in the previous report,[1] and will again be observed here.[2]

A. Monoterpenes

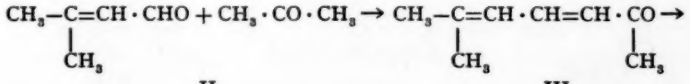
In the reaction of small quantities of sulphuric acid on a solution of isoprene in glacial acetic acid, Wagner-Jauregg observed the formation of geraniol, α-terpineol, and 1,4- as well as 1,8-cineol. Inferences from this observation as to the origin of terpenes in nature can be made only with strong reservations.

Cahn, Penfold & Simonsen isolated the previously unknown dehydrogeranic acid (Formula I) by saponification of its geranyl ester, which occurs in the wood of *Callitropsis araucarioides*. The constitution of this acid was established by oxidation to acetone and catalytic hydrogenation to tetrahydrogeranic acid. Strong evidence



I. Dehydrogeranic acid

for the correctness of the formula was furnished by the synthesis simultaneously carried through by Kuhn & Hoffer and by Fischer & Löwenberg in which 2-methylbuten-(2)-al-(4) (Formula II) was condensed with acetone, and the resulting ketone (Formula III) converted with bromoacetic ester and zinc. After loss of water from the hydroxy ester IV and saponification, dehydrogeranic acid was obtained.

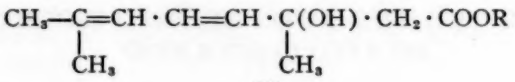


II III

* Received January 29, 1934.

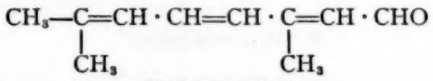
[1] *Ann. Rev. Biochem.* 1, 581 (1932)

[2] This review embraces such works as are referred to in *Chemisches Zentralblatt* from December, 1931, to September, 1933.



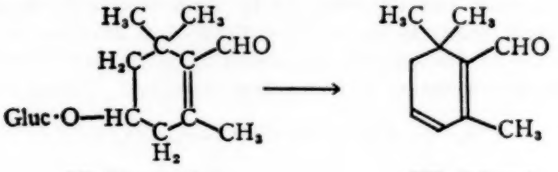
IV

Still more noteworthy than this synthesis is the observation of Fischer & Löwenberg that the aldehyde II may be condensed with barium hydroxide to dehydrocitral (Formula V).



V. Dehydrocitral

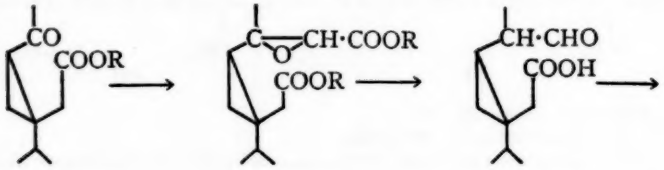
Kuhn & Winterstein have thrown light upon the closely related active taste-princple (*Geschmackstoff*) of saffron, picrocrocine, which apparently possesses Formula VI and on hydrolysis is broken down into glucose and the still-unknown safranal (Formula VII).



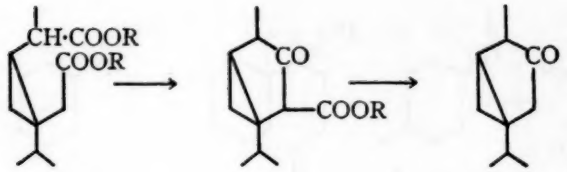
VI. Picrocrocine VII. Safranal

Kaku, Cho & Orita have identified eucarvone in a variety of *Asarum Sieboldii*. This is the first observation of a monocyclic seven-membered ring in nature.

Ruzicka & Koolhaas describe a partial synthesis of thujone (Formula XIII), proceeding from its degradation product, α-thujaketo acid. The ester (Formula VIII) of the latter was converted by way of the glycide ester IX into the aldehyde acid X. The dicarboxylic ester XI prepared from this gave, upon treatment with sodium, the keto ester XII, which yielded thujone after the ketone cleavage.



VIII IX X

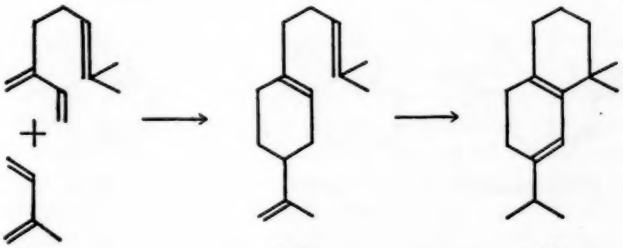


XI XII XIII. Thujone

The interconversion of optically active systems of the camphor series, which was more fully described in the previous report, has been further pursued by Houben & Pfankuch.

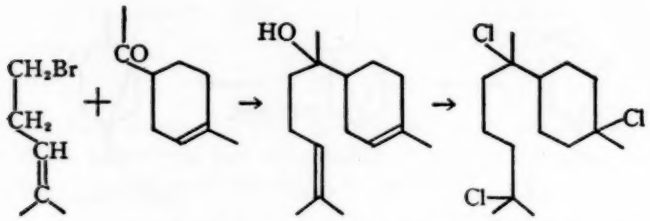
B. Sesquiterpenes

Connected with the above-mentioned work of Wagner-Jauregg is the addition of isoprene to myrcene, investigated by Ruzicka & Bosch. In this, corresponding to the polymerization of isoprene to dipentene, there is formed the monocyclic sesquiterpene XIV, which upon treatment with formic acid passes over into the bicyclic hydrocarbon XV. These formulae are supported by the dehydrogenation of XV to eudaline (1-methyl 7-isopropyl-naphthalene).



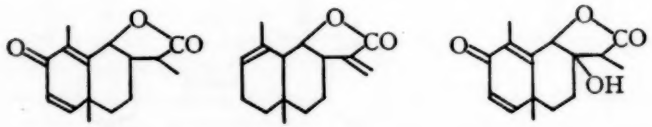
XIV XV

Bisabolene, the monocyclic sesquiterpene most widely distributed in nature, was synthesized by Ruzicka & Liguori. The synthesis, the first not depending on rearrangements, was accomplished by the reaction of the magnesium compound of 2-methyl-5-bromopentene-(2) (Formula XVI) with 1-methyl-4-acetyl-cyclohexene-(1) (Formula XVII). The monocyclic sesquiterpene alcohol XVIII, so obtained, yielded with HCl the trihydrochloride of bisabolene (Formula XIX).



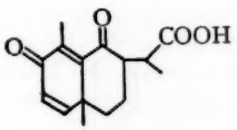
XVI XVII XVIII XIX. Bisabolene trihydrochloride

In the field of the lactones of the sesquiterpene series (see also the previous report), lie further investigations by Ruzicka, Pieth, Reichstein & Ehmann, as well as by Hansen, on the alantolactones, which, however, have brought as yet no complete elucidation of their constitution. It appears to be established that, despite their crystalline nature, these lactones represent mixtures of isomers with different positions of the double bonds, as they ordinarily occur in liquid terpene mixtures. In any case, it is established that here we are dealing with compounds (see Formula XXI as an example of one of the alantolactones) which show the same structure as santonine (Formula XX).

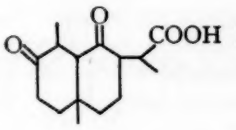


XX. Santonine XXI XXII

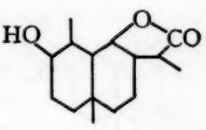
According to investigations by Tettweiler, Engel & Wedekind, artemisine belongs to the same group, and, on the basis of the following conversions, is recognized as hydroxysantonine (Formula XXII). Boiling XXII with sodium hydroxide leads to the formation of the diketo acid XXIII through loss of water from the dihydroxy keto acid, assumed as an intermediate product. By catalytic hydrogenation of XXIII one obtains the saturated product XXIV, which can also be prepared from XXII by catalytic hydrogenation and subsequent boiling of the tetrahydro-artemisine with sodium hydroxide. Reduc-



XXIII



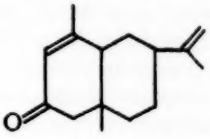
XXIV



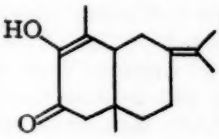
XXV

tion of both keto groups of XXIV by means of sodium amalgam results in the formation of XXV, which is identical with the hexahydro-santonine obtained through catalytic hydrogenation of santonine.

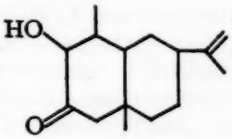
Through work of Bradfield, Penfold & Simonsen, ketones of a new type in the sesquiterpene series have become known. From the wood oil of *Eremophila Mitchelli*, three compounds with the very probable Formulae XXVI, XXVII, XXVIII have been isolated. Eremophilone (XXVI) yields a hydroxy methylene compound, and



XXVI. Eremophilone

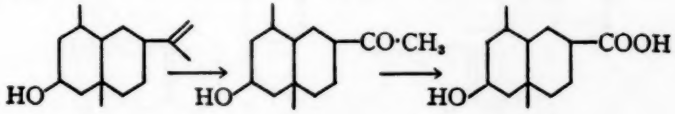


XXVII

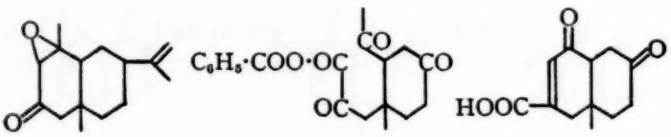


XXVIII

can be hydrogenated to a tetrahydro derivative. Sodium and alcohol give the carbinol XXIX, from which, upon heating with selenium, one obtains eudaline. Action of ozone on XXIX results in the hydroxyketone XXX, whose oxidation with sodium hypobromide leads to the hydroxy acid XXXI. Treatment of XXVI with hydrogen peroxide in alkaline solution yields the oxide XXXII, from which can be prepared the same benzoyl derivative as from XXVII. The breakdown of the benzoate of XXVII with ozone leads to the establishment of the position of the double bonds in XXVII, for there results from this, through ring-closure and saponification of the primarily-formed XXXIII, the diketo acid XXXIV.



XXIX XXX XXXI

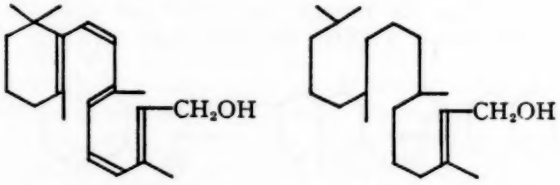


XXXII XXXIII XXXIV

In the compounds XXVI–XXVIII there indeed occur mixtures of isomers with different positions of the individual double bonds. On ozonization, at any one time only one of these yields characteristic degradation products. Thus XXVIII gave, with ozone, formaldehyde and the expected hydroxy diketone.

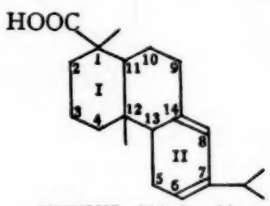
C. Diterpenes

Considerable progress has been made recently in the chemistry of the diterpenes. In the first rank belongs the elucidation of the constitution of vitamin A (Formula XXXV) by Karrer, which is further discussed in the section on vitamins. There is present in vitamin A the same chain, regularly built up from four isopentane residues, as in phytol (Formula XXXVI), the alcoholic constituent of chlorophyll cleared up some years ago by Fischer. Both of these alcohols show schematic half-structures of the carbon skeleton of the tetraterpene carotene, which consists of two symmetrical halves corresponding to vitamin A or phytol. These results demonstrate the uncommonly marked distribution in nature of the synthesis principle (*Aufbauprinzip*) of the terpenes. We wish to emphasize that the same carbon structure, as is contained in XXXV and XXXVI, probably also belongs to some "typical" representatives of the diterpene series, such as agathene di-acid and sclareol, on which a few details were communicated in the previous report.

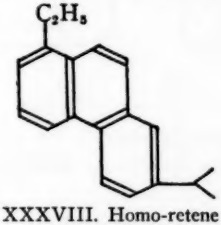


XXXV. Vitamin A XXXVI. Phytol

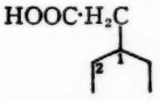
Important progress is recorded in the elucidation of the carbon structure of the most important representative of the true diterpenes, abietic acid, leading to advancement of Formula XXXVII. The positions assigned to the double bonds, though not yet proved with complete certainty, may, however, be regarded as exceedingly probable. The difference between the carbon structure of Formula XXXVII and the carbon structure hitherto assigned to abietic acid on the basis of older work lies in the location of the carboxyl, for which previously one of the positions 2, 3, or 4 was assumed. In 1922 Ruzicka & Meyer accomplished the reduction of abietic acid ester with sodium and alcohol to abietinol, and the loss of water from the latter. The hydrocarbon so obtained, which thus should contain a methyl group in the place of the carboxyl, was dehydrogenated, by which means was obtained a homo-retene, $C_{19}H_{20}$, for which the constitution of a methyl-retene was considered, with the location of the new methyl group in 2, 3, or 4. Ruzicka, de Graaff & Müller now obtained by the oxidation of the homo-retene with potassium ferricyanide the same phenanthrene-1,7-dicarboxylic acid, that was formed by similar breakdown of retene. This indicates for homo-retene the constitution of 1-ethyl-7-isopropyl-phenanthrene (Formula XXXVIII). The type of bond for the carboxyl group of abietic acid should accordingly be that of Formula XXXIX. Ruzicka, Waldmann, Meier & Hösli, however, were able to show that this is not the case, but that with the loss of water from abietinol (Formula XL) a pinacoline rearrangement occurs to give hydrocarbon XLI. One can avoid the latter rearrangement by first oxidizing XL to abietinal (Formula XLII), and reducing its semicarbazone to hydrocarbon XLIII by heating with



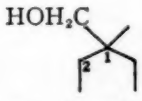
XXXVII. Abietic acid



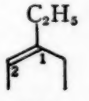
XXXVIII. Homo-retene



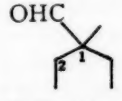
XXXIX



XL. Abietinol



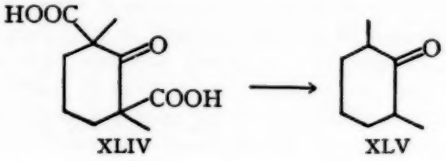
XLI



XLII. Abietinal

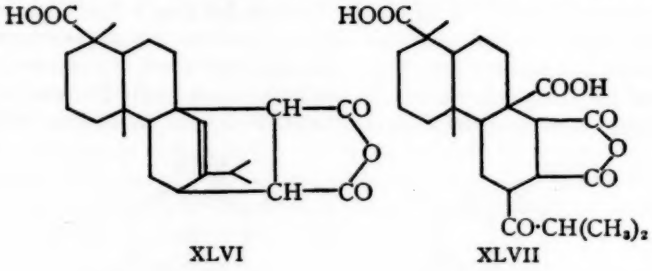
XLIII

sodium ethylate. On dehydrogenation of the latter with selenium there is formed not homo-retene but retene. A further confirmation of the structure of ring I of abietic acid was given by the formation, observed by Ruzicka & Waldmann, of 1,3-dimethyl-cyclohexanone-(2) (Formula XLV) upon oxidation of abietic acid with potassium permanganate, which proceeds probably by way of the intermediate product XLIV.

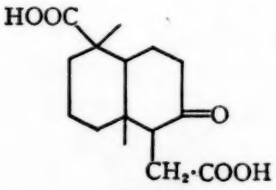


XLIV XLV

On the basis of earlier investigations it was already established that both double bonds of abietic acid must be in ring II. The location 9,14 could also be considered for one of them. When Ruzicka, Ankersmit & Frank, as well as Arbusow, carried through the addition of maleic acid anhydride to abietic acid, obtaining an addition compound (Formula XLVI), the possibility of a double bond in 9,14 was eliminated on stereochemical grounds. Formula XLVI and hence also the location of the double bond according to Formula XXXVII are



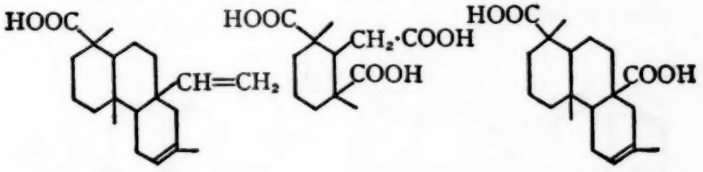
XLVI XLVII



XLVIII

favored by the results of Ruzicka, Waldmann, Meier & Hösli on the action of ozone on XLVI as also on abietic acid ester. In the first case there was obtained a compound of the composition represented by Formula XLVII, and in the latter, one possessing that of Formula XLVIII.

On the basis of investigations by Ruzicka, de Graaff, Goldberg & Frank, as well as by Ruzicka, de Graaff & Müller, there may be suggested for the second important resinic acid, dextro-pimaric acid, Formula XLIX, in which only the location of the double bond in ring II is not established with entire certainty; it is certain, however, that this double bond can be only in ring II. In an analogous manner to abietic acid, dextro-pimaric acid yields 1-ethyl 7-methyl phenanthrene after loss of water from the dextro-pimarol prepared by reduction of the ester, and after dehydrogenation of the hydrocarbon thereby formed. Oxidation of XLIX with nitric acid yielded the acid L, also obtained from abietic acid. Finally, the dihydroxy acid obtained by the partial oxidation of XLIX with potassium permanganate yielded upon further breakdown with chromic acid the dicarboxylic acid LI, which, like dextro-pimaric acid itself, can be dehydrogenated to pimanthrene (1,7-dimethyl-phenanthrene), and upon hydrogenation yields a dihydro acid. The formation of formaldehyde on ozonization of XLIX corresponds to the presence of the vinyl group. It is worthy of note that the carbon skeletons of For-

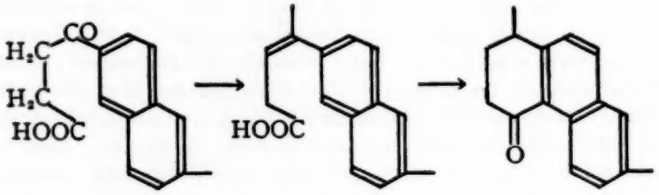


XLIX. Dextro-pimaric acid L LI

mulae XXXVII and XLIX, in contrast to the diterpenes mentioned in the introduction, consist not of a regular, but of an irregular isoprene chain.

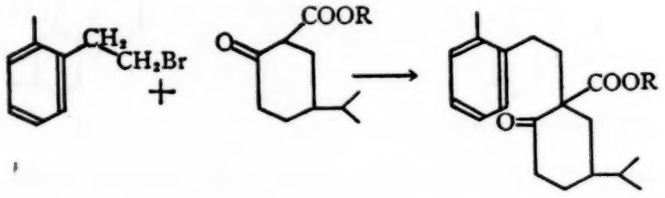
There has been substantial progress in the elucidation of the constitution of XXVII and XLIX through the synthesis of the phenanthrene hydrocarbons, pimanthrene and retene, which as mentioned above may be prepared from XXXVII and XLIX. This was accomplished simultaneously by Haworth, Letsky & Mavin, and by

Bardhan & Sengupta (1, 2). Haworth has also prepared synthetically the ethyl compound, XXXVIII, and the 1-ethyl-7-methylphenanthrene. Both types of synthesis may indeed be illustrated by a single example. Haworth and co-workers, for instance, converted β-methyl naphthalene with succinic acid anhydride to LII; from this they prepared LIII with methyl magnesium iodide, which after hydrogenation and ring closure yielded LIV. From the latter, after reduction of the keto group and dehydrogenation, pimanthrene was obtained.



LII LIII LIV

Bardhan & Sengupta condensed β-*o*-tolyl-ethyl-bromide (Formula LV) with isopropyl-cyclohexanone-carboxylic-ester (Formula LVI) to LVII; in this they reduced the keto group to a hydroxy group, closed the ring with phosphorus pentoxide, and dehydrogenated to retene with selenium.

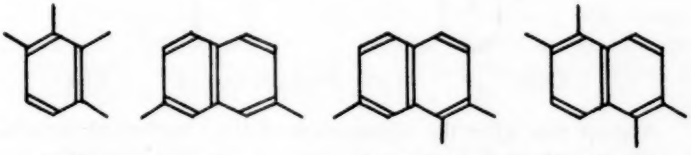


LV LVI LVII

Moreover, Haworth & Mavin also synthesized 1,7,8-trimethyl phenanthrene, whose preparation from isoagathene dicarboxylic acid was mentioned in the previous report, strongly supporting the carbon structure therein assigned to agathene di-acid.

D. Triterpenes and Sapogenines

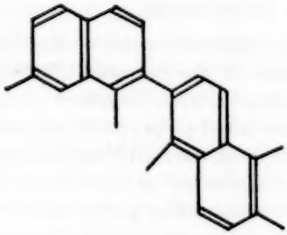
While it was possible in the previous review[1] to report on the elucidation of the constitution and synthesis of the aliphatic triterpene squalene, until recently there was, in general, no information of any kind on the structure of the widely distributed polycyclic triterpenes, which as a rule contain five rings, or on that of the related sapogenines. The fortunate experiences with dehydrogenation in elucidating the constitution of the sesquiterpenes and diterpenes also gave to dehydrogenation the center of interest in studies on the triterpenes. By heating with selenium, different representatives of the triterpene and sapogenine series have been dehydrogenated, in particular amyrine, betuline, hederagenine, oleanolic acid, suma-resinolic acid, sia-resinolic acid, and gypsogenine (cf. Ruzicka, Brüngger, Egli, Ehmann, Furter & Hösli; Ruzicka, Silbermann & Pieth; Ruzicka, Brüngger, Egli, Ehmann & Goldberg; Späth & Hromatka; Brunner, Hofer & Stein). By this means a series of aromatic dehydrogenation products have been isolated of which only the compounds best characterized and to some extent already completely elucidated will be listed here. These are 1,2,3,4,-tetramethyl-benzene (Formula LVIII), 2,7-dimethyl-naphthalene (Formula LIX), 1,2,7-trimethyl-naphthalene (sapotaline) (Formula LX), hydroxy sapotaline (with the location of the hydroxyl group still unknown), dinaphthyl compounds of the probable empirical formula $C_{22}H_{24}$ (cf. the hypothetical formula LXII) or $C_{27}H_{28}$, and finally a hydrocarbon $C_{25}H_{20}$, probably trimethyl-picene (hypothetical formula LXIII).



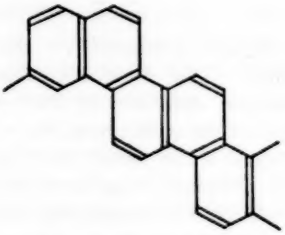
LVIII LIX LX. Sapotaline LXI

The hydrocarbons LVIII to LXI are admirably elucidated by identification with synthetic compounds. The synthesis of sapotaline has been described by Ruzicka & Ehmann and that of the hydrocarbon LXI by Ruzicka, Ehmann & Mörgeli. These results lead to Formula LXIV for the carbon structure of the pentacyclic triterpenes, which

[1] *Ann. Rev. Biochem.*, 1, 581.

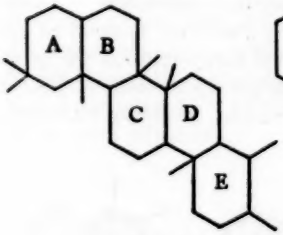


LXII

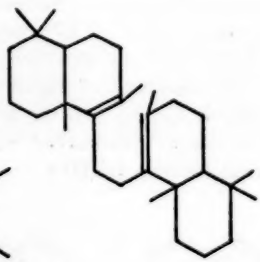


LXIII. Trimethyl-picene (?)

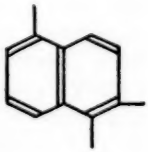
is to be regarded only as a working hypothesis. This carbon skeleton can be broken down into six isoprene residues, and is closely related in structure to tetracyclo-squalene (Formula LXV). The formation, mentioned in the previous report, of 1,2,5-trimethyl naphthalene (Formula LXVI) upon dehydrogenation of LXV with selenium gave rise to the assumption of analogous relationships between compounds LX, LXI, and LXIV.



LXIV



LXV. Tetracyclo-squalene



LXVI

A short time after the advancement of the hypothetical formula LXIV, Rosenheim & King, on the basis of the dehydrogenation results of Diels, proposed for cholesterol a formula[3] which shows a certain similarity to the structure LXIV. The method of elucidating the constitution of the sterols and bile acids through systematic decomposition, with complete disregard of the results of dehydrogenation, as followed particularly by Wieland and Windaus, was unusually difficult, and productive of errors in principle, and for

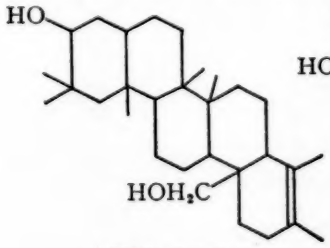
[3] Cf. the section on sterols and bile acids, page 87.

this reason attempts to adopt the reverse procedure in the triterpene series are justified. From this point of view, a few of the more important results in the investigation of triterpenes and sapogenines are compiled below.

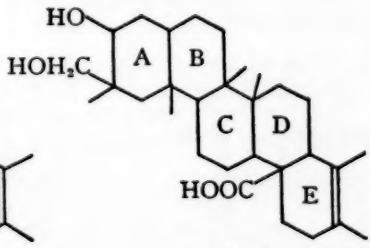
Ruzicka & Furter, as well as Winterstein & Stein, were able to show that different sapogenines, as for example hederagenine and oleanolic acid, to which formulae with thirty-one carbon atoms were formerly assigned, on the basis of careful micro-analyses and especially titrations, have only thirty carbon atoms, and therefore are properly to be assigned to the triterpenes.

The most natural polycyclic triterpenes would contain six rings if they possessed no double bond. Previously, only in exceptional cases, as for example in betuline, could a double bond be proved through catalytic hydrogenation. In the previous paper there was mentioned a further method discovered by Winterstein—the formation of bromolactones by the addition of bromine to triterpene acids. This was recently employed by Winterstein & Egli with success on sia- and sumaresinolic acids. Ruzicka, Silbermann & Furter were able to demonstrate the pentacyclic structure of amyrine through employment of molecular refraction and the determination of the oxygen number by means of perbenzoic acid.

Betuline was recently studied by Ruzicka, Brüngger & Gustus, as well as by Vesterberg. On the basis of the known behaviour of this triterpene-diol, and employing the hypothetical structure LXIV, Ruzicka, Brüngger, Egli, Ehmann, Furter & Hösli proposed Formula LXVII for it. This particularly explains why betuline passes over into a saturated isomeric hydroxy oxide (allobetuline) with disappearance of the functions of the primary alcohol group and the double bond. Also it further explains the fact that in allobetulone a CH_2 group still remains near the CO group.

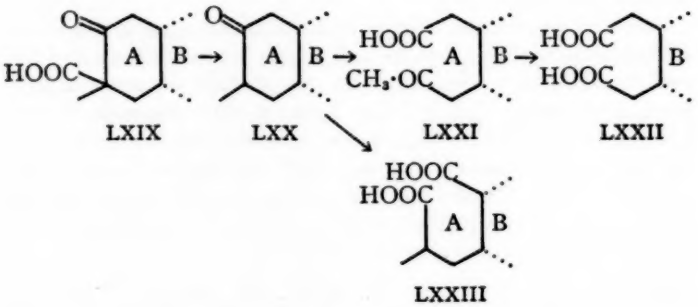


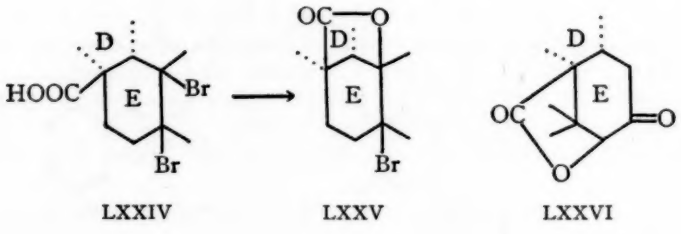
LXVII. Betuline



LXVIII

Still more exhaustive results are presented in the field of hederagenine and oleanolic acid, where the investigations of earlier workers, now particularly of Kitasato & Sone, were so fruitful. Here also Formula LXVIII proposed by Ruzicka, Brüngger, Egli, Ehmann, Furter & Hösli forms, in principle, a useful basis for explaining the transformation of hederagenine. A formula for oleanolic acid follows from Formula LXVIII after substitution of the primary hydroxyl by hydrogen. The similar position of the secondary hydroxyl in LXVII and LXVIII results from the formation of the same hydroxy-sapotaline on dehydrogenation. The 1–3 position of the two alcoholic hydroxyls in Formula LXVIII explains well the observation by Jacobs & Gustus of several years ago that carbon dioxide splits off in the oxidation of the primary hydroxyl group to carboxyl and of the secondary to a keto group, for through this a β-keto acid (compare the transformation LXIX to LXX formulated for ring A) must appear as an intermediate product. The opposite positions of the carboxyl and the double bond in LXVIII are in accord with the formation of a bromolactone observed by Winterstein upon bromine addition (compare the formulation of the change LXXIV to LXXV using ring E).





The oxidation of LXX with potassium hypobromide, on the one hand through LXXI to LXXII, on the other to LXXIII, observed by Kitasato & Sone, can likewise be well formulated. At present, a lack of agreement exists only over the structure of ring E, for Kitasato & Sone describe a keto-lactone (Formula LXXVI, ring E) from it, whose formation would infer another type of distribution of the methyl groups in E. However, since such a distribution of the methyl groups no longer harmonizes with Formula LXI for tetramethyl naphthalene, further investigation is required to clear up the confusion on this point.

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THE BIOCHEMISTRY OF THE NITROGENOUS CONSTITUENTS OF THE GREEN PLANTS*

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The field covered in this review is restricted to papers published during the past two years that deal with nitrogenous substances isolated from, or detected in, green plant tissue, and with the changes that take place in the proportions of such substances during the life of the plant.

The amide asparagine has long been recognized as an important constituent of plants (see Prjanischnikow), but its next higher homologue, glutamine, has only seldom figured in the literature. Chibnall & Westall have developed a method whereby glutamine may be detected and determined in the presence of asparagine, and have employed it in a brief study of extracts of seedlings of *Ricinus communis, Brassica oleracea, Helianthus annuus,* and *Vicia faba,* and of the roots of *Apium graveolus.* Both glutamine and asparagine were detected in each case. Etiolated seedlings of *Ricinus* (castor bean) contained nearly four times as much glutamine as asparagine, the two amides together accounting for two-fifths of the water-soluble nitrogen. The roots of *Apium* (celery) were also exceptionally rich in glutamine. The amount of glutamine found in broad bean seedlings was so small as to be within the limits of error of the method.

Kitagawa & Yamada have continued the study of the basic substance $C_5H_{12}O_3N_4$ previously isolated by Kitagawa & Tomita from the jack bean. They find that it is hydrolyzed by liver extracts with the production of urea and of a diamino acid $C_4H_{10}O_3N_2$, and therefore believe that the base, for which they suggest the name canavanine, is a guanidine derivative.

Sasaki has subjected an extract from germinated soy beans to detailed analysis and has isolated asparagine, adenine, guanine, histidine, arginine, choline, and cadaverine; lysine was not found.

Schmalfuss, Heider & Winkelmann have investigated aqueous extracts of soy beans for tyrosine and dihydroxyphenylalanine. The separation of these closely allied substances was effected by precipitating dihydroxyphenylalanine from a faintly alkaline solution with

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basic lead acetate, and subsequently throwing down the tyrosine at a more strongly alkaline reaction with the same reagent. The products were recovered, after decomposition of the precipitates with hydrogen sulfide, by crystallization at neutral reaction.

Dihydroxyphenylalanine has attracted considerable attention because of its relation to adrenaline. Syntheses are described by Deulofeu & Mendivelzua and by Heard.

Nelson, Mottern & Eddy have examined the juice of Florida valencia oranges for simple nitrogenous substances. They identified stachydrine, arginine, choline, asparagine, and aspartic acid together with a mixture of mono-amino acids from which pure individuals were not isolated.

Kishi has attempted to determine cystine and cysteine in deproteinized extracts of mulberry leaves by the iodine-titration method of Okuda. The substance which reacts with iodine increases in amount with the age of the leaves and reaches a maximum at the stage where synthetic action is most vigorous. Whether or not the reactive substance is really cystine, however, requires demonstration by a more specific method. In this connection the work of Kozlowski (1) should be recalled. This author some years ago obtained what were almost certainly crystals of cystine, although in minute yield, from an extract of peas [see also Kozlowski (2)].

Evans has analyzed pasture grass for cystine. Traces only could be obtained by direct isolation methods, although the Folin colorimetric method suggested the presence of appreciable amounts. The more specific Sullivan method, however, indicated that the proportion was very small, and methods that depend on determination of sulfide sulfur indicate that it is less than 0.1 per cent of the dry weight. In view of these findings it seems difficult to account for the cystine in the wool of animals fed such pasture grass unless synthesis from some precursor in the grass is possible in the animal body.

Klein & Tauböck (1) have investigated the problem of arginine metabolism in the higher plants. They employed an adaptation of the quantitative method of Weber, which is founded upon the Sakaguchi color reaction. The only interfering substances that are likely to be encountered in plant extracts are galegine, which has hitherto been found only in *Galega officinalis* (Klein & Schlögl), and agmatine, found only in certain molds. Citrulline and canavanine do not give the reaction.

The free arginine was determined in extracts from the tissue,

total arginine was determined in samples of tissue that had been subjected to hydrolysis with acid under pressure, and the combined arginine was ascertained by difference. Etiolated seedlings of leguminous plants showed a continuous increase with age in free arginine at the expense of the protein arginine. The total arginine, however, diminished. Evidence was found that free arginine was in part used up in metabolic reactions. Some of the arginine so used was probably decomposed to urea, although direct evidence on this point was not obtained.

The arginine metabolism of conifer seedlings (*Pinus pinea*) was entirely different from that of the legumes. Etiolated pine seedlings showed little or no utilization of arginine, but, in a remarkably short space of time, nearly the whole of the arginine of the seed (originally 94 per cent combined) was liberated in the free form. Normal pine seedlings, studied over a period of some 60 days, showed little evidence of arginine synthesis. Although the total protein increased more than 100 per cent, the amount of combined arginine remained at a constant low level. Conifers behave in this respect much like other plants—arginine synthesis first becomes of importance when seed ripening takes place.

The formation of urea in seedlings cannot in all cases be wholly accounted for in terms of arginine decomposition. Klein & Tauböck (1) point out the possibility that some substance, such as Kitagawa & Tomita's canavanine, may play a part. In this connection, however, attention should be directed to the work of Krebs & Henseleit, who have obtained evidence that urea formation in the animal body involves a series of reactions in which carbon dioxide and ammonia react with ornithine to produce citrulline, this in turn combines with more ammonia with the formation of arginine, which then is decomposed, presumably by arginase, to ornithine and urea. Ornithine therefore assumes the rôle of a catalyst, and citrulline, which was originally discovered in a plant tissue by Wada, that of an intermediate. It is quite possible that some such series of reactions may take place in plant tissues.

Klein & Tauböck (2) have also studied the behavior of sterile cultures of *Zea mays* and *Phaseolus vulgaris* in which arginine was added to the culture solution. Maize is a typical example of a plant that does not contain urea, whereas the bean plant contains appreciable amounts. Analyses of the culture solutions showed that arginine was rapidly taken up by the roots of both species without apparent

decomposition; the solution from a spontaneously infected culture contained both urea and ornithine. A marked increase of free arginine was found in the bean plants, particularly in the roots; there was also an increase in urea nitrogen both in the roots and in the green parts. The increase in free arginine and in urea by no means accounted for the arginine removed from the culture solution; it was fairly clear that most of the absorbed arginine entered directly into metabolic reactions. Similar results were secured with maize, although no accumulation of urea occurred.

The arginine metabolism of leaves defective in chlorophyll has been studied by v. Euler & Burström. They employed the technique of Klein & Tauböck (1). No essential difference in the content of free arginine was found between chlorophyll-defective mutants of barley and the normal plants; chlorophyll therefore bears no close relation to that part of the nitrogen metabolism which has to do with arginine. On the other hand the colorless parts of variegated *Pelargonium* leaves contained fifty to one hundred times as much free arginine as the green parts. No conclusions were drawn from this observation, and the study of variegated leaves is to be continued.

Klein & Linser (1) have developed a method whereby choline, both in the free form and also that combined in lecithin, can be determined in small samples of plant tissue. The method depends on the decomposition of choline, when heated with 33 per cent sodium hydroxide, to give trimethylamine; this is distilled off in a special apparatus, and the distillate is treated with a few drops of 33 per cent hydrogen peroxide to destroy aldehydes that may be present. After the lapse of a proper time interval, the distillate is transferred to a Parnas-Wagner apparatus and the volatile base is distilled. Ammonia is determined in a part of the distillate by Nessler's method with the aid of a Pulfrich photometer (Zeiss), and the total base is determined by titration; the quantity of trimethylamine is obtained by difference. Lecithin choline is determined in a similar manner after decomposition of the lecithin with 50 per cent potassium hydroxide. Extracts of plant tissues are prepared by maceration at room temperature with 96 per cent alcohol; the extract is evaporated *in vacuo,* dissolved in 20 cc. of water, and extracted with 100 cc. of ether for one hour. After being allowed to settle, as much ether as possible is decanted and the aqueous layer is treated with a little sodium chloride and centrifuged. The thin layer of ether that remains is allowed to evaporate and 15 cc. of the clear aqueous fluid are with-

drawn. Choline is estimated in this, and the amount found is increased by one-third to give the choline in the whole of the aqueous fluid. The ether extract (lecithin extract) is evaporated, the remaining 5 cc. of aqueous fluid are added, and the lecithin choline is determined. The lecithin choline is calculated by subtracting an amount equivalent to the 5 cc. of aqueous fluid, and is further corrected for the solubility of free choline in wet ether, data for which are provided.

Klein & Linser point out that this method really determines all of the stable volatile base that can be liberated by treatment with alkali. They believe that by far the greater part of the volatile base consists of trimethylamine derived from choline—a correction for ammonia is specifically provided. Nevertheless it is possible that trimethylamine may also arise from the decomposition of betaines present in the plant tissue. They propose to avoid this difficulty by selection of suitable plant material. In any case the decomposition of these substances is not quantitative and the betaines are thought to be present in small proportions relative to the choline. How well founded the assumptions implied in this method may be remains to be demonstrated.

Data are presented for the free and lecithin choline per individual plant at various stages during germination both in light and in the dark. The quantity of choline found varied between 0.01 and 0.10 per cent of the fresh weight in most cases; the seeds and seedlings of leguminous plants, however, contained even larger proportions. Etiolation of the seedlings increased the free choline and depressed the lecithin choline. Investigations of plants during the whole vegetative period were also conducted. The behavior of the two forms of choline was such as to suggest that lecithin was the end product and free choline the intermediate in the metabolism of the two substances. Lecithin choline was found to migrate from organ to organ of the mature plant, that is, it may be withdrawn from the leaves and stored in the stem or *vice versa*. More choline and lecithin choline are formed during fruiting than are present in the flowers; migration of these substances must therefore take place.

Klein & Linser (2) have also studied the metabolism of the betaines stachydrine and trigonelline. The method employed consists of extracting 1 to 10 gm. of the dry tissue with 1 per cent hydrochloric acid at room temperature. Aliquot parts of the concentrated extract are treated with potassium bismuth iodide reagent and iodine. The precipitate is filtered off, dissolved in warm dilute sodium hy-

droxide, and then acidified with sulfuric acid. A small amount of sodium sulfite is added to prevent the separation of iodine, and 20 per cent potassium iodide solution is added in excess. The yellow solution obtained is suitably diluted and the concentration of bismuth in it is determined by means of the Pulfrich photometer; the stachydrine or trigonelline content of the original extract is then calculated by means of the proper factors.

A study of seedlings of *Trigonella foenum-graecum* showed that an accumulation of trigonelline took place from the sixth to fifteenth days when the seedlings were etiolated, and the level attained was appreciably higher than that in normal seedlings.

An extensive series of experiments was conducted in which amino acids were administered to growing plants. On purely chemical grounds it seemed probable that proline, ornithine, and glutamic acid might stimulate the formation of stachydrine and trigonelline. Several techniques, among them the vacuum infiltration method of Björkstén, were employed, the most successful being injection of the amino acid in solution into the hollow stems of the plants. *Stachys palustris,* treated in this way with proline, showed marked increases in the stachydrine content of flowers and leaves; *Dahlia variabilis* responded with similar increases in trigonelline. Similar results were secured by injection of ornithine, and the increase in the betaine was even more marked when urotropine was added to the injected solution to provide a source of formaldehyde. No increase in betaine content could be established after the injection of glycine, alanine, leucine, tyrosine, arginine, aspartic acid, asparagine, citrulline, or ammonium nitrate.

A study of the trigonelline content of several plants throughout their life showed that the quantity of this substance increased at some stages and decreased at others, in other words trigonelline is not only synthesized but is also utilized; it is therefore not simply an end-product of metabolism.

Klein, Krisch, Pollauf & Soos have applied a microchemical method to the detection of trigonelline and stachydrine in numerous plants and have greatly extended the knowledge of the distribution of these substances.

Klein & Linser (3) have studied the effect of culture solutions that contained proline upon the nicotine content of tobacco plants. The whole plant was cut and the stem was placed in the solution. Their data indicate an increase in nicotine content over the control

plants under these conditions. They consider that proline can behave as the precursor not only of the pyrrolidine ring but also of the pyridine ring of nicotine.

Nottbohm & Mayer (1) have detected the presence of trigonelline and choline in coffee. They (2) have also demonstrated choline in the seeds and leaves of the tobacco plant.

Fosse, de Graeve & Thomas have investigated the seeds of a wide variety of plants for uric acid. Quantities of the order of 0.025 per cent (*Melilotus officinalis*) were found in a number of grains and in certain legumes, smaller proportions in other legumes. Uric acid was isolated and positively identified in some cases. The occurrence of uric acid in seeds accounts for the presence of the enzyme uricase, and it is probable that uric acid is a precursor of the allantoin long known to be widely distributed in seeds.

Although the discussion of alkaloids is reserved for another reviewer, attention should be directed here to the paper by Cromwell on the function of alkaloids in plants. A thorough study of the distribution of berberine in *Berberis Darwini* has convinced this investigator that the alkaloid is definitely an end-product of metabolism. Accumulation takes place through the summer but no loss supervenes in winter. Fertilization with asparagine increased the alkaloid content. Growth on a nitrogen-free culture medium did not reduce the alkaloid content; on the other hand the normal increase with age occurred. Etiolation leads to the production of shoots rich in berberine. If the bark is ringed the shoots appear only below the ring, suggesting that berberine is synthesized from products translocated from the roots. But ringed plants grown in light show an accumulation of berberine above the ring, suggesting synthesis from products derived from the leaves. The conclusion is drawn that berberine is an end-product of protein metabolism the synthesis of which involves condensation with substances derived from the carbohydrate metabolism.

Studies of various phases of the metabolism of the tobacco plant have appeared from several laboratories. Eisenmenger has studied the nitrate nitrogen and amino nitrogen of plants grown in water culture under conditions of bountiful supply and deprivation of nitrate. Parallel experiments were conducted in light and in dark. All the plants maintained a high level of nitrate nitrogen in the tissue, those grown in the dark containing more than those grown under normal conditions of illumination. The plants to which ample nitrate, but a disproportionally low supply of nitrogen in other forms, had been

furnished contained a lowered proportion of organic solids, and a high proportion of nitrate, indicating a lowered rate of metabolism.

Beaumont, Larsinos, Piekenbrock & Nelson have studied the assimilation of various substances from sterile water culture by tobacco plants. Nitrate was most readily absorbed; urea, ammonium salts, asparagine, and cystine were taken up to a smaller extent, but other amino acids were not absorbed.

Vickery, Pucher, Wakeman & Leavenworth have studied the chemical changes that take place in the leaves of Connecticut shade-grown tobacco when these are subjected to curing under normal conditions, and also when the leaves are placed with their cut bases in distilled water. A rapid increase in the amount of nitrate nitrogen occurred in both cases. This can be explained only on the assumption of a synthesis of nitrate in the leaf tissue. The subsequent behavior of the nitrate in the two cases was entirely different. No diminution in quantity occurred in the later stages of curing, but a transformation of much of the nitrate, apparently to ammonia, took place in the later stages of water culture. The quantity of nicotine in the curing leaves at first increased slightly and then diminished. This was interpreted as an indication that nicotine shares in the metabolism in some manner other than as a waste product. Synthesis did not occur, however, in the cultured leaves, although a slow diminution in quantity took place. Rapid digestion of the protein to products soluble in hot water occurred in both cases, together with parallel increases in the proportion of amino, ammonia, and amide nitrogen. Evidence was secured that carbohydrate was metabolized in the water-cultured leaves in preference to protein. Further support was obtained for the view of Prjanischnikow that the mechanism of amide synthesis, in particular that of asparagine, is provided as a protective measure against the accumulation of ammonia in the plant tissues. This observation, made upon mature leaves, is a confirmation of the earlier work of Mothes (1).

The broad outlines of the nitrogen metabolism of a number of different plants have formed the theme of a series of papers from different laboratories. Platenius has worked with the celery plant, Knowles & Watkin with wheat, Wagner with oats, and Mothes with *Syringa, Nicotiana,* and *Phaseolus* species. Mulay has studied seasonal changes in the composition of the non-protein nitrogen in the shoots of the pear, and Culpepper & Caldwell have dealt with the relation of

age and of seasonal conditions to the composition of the rhubarb plant.

A definite trend is shown, in many of the papers that deal with various aspects of metabolism, toward the expression of analytical results upon a biological rather than on a strictly chemical basis. Instead of dealing with, for example, nitrate nitrogen expressed as a percentage of the dry or fresh weight, investigators are beginning to appreciate the desirability of expressing their results in terms of a biological unit, e.g., the weight per plant, or per definite leaf area, or weight of metabolizing tissue. Biological relationships are thereby made more vivid, and the services that chemistry can give to plant physiology are being more thoroughly exploited. It is also to be noted that analytical methods are being widely and critically examined with a view to improving their accuracy and applicability.

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MINERAL NUTRITION OF PLANTS*

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The main chapter of mineral nutrition deals with the general laws of the uptake of plant food by the roots. Permeability generally means the entrance of dissolved substances into the cell. But from a wider point of view permeability is only a single stage in the great process of the uptake and distribution of solutes in the whole organism. Recently there has been an increased number of publications that attack the problem by accurate chemical and physical methods instead of the more qualitative permeability method. Among suitable analytical methods that of quantitative spectral analysis (25) has rendered good service, especially for the thorough study of cation nutrition. There is also an increasing tendency to apply the results and viewpoints of colloidal and physical chemistry to the problems of mineral nutrition.

Salt absorption as a problem of colloidal chemistry.—The absorption of non-electrolytes by the cell is the main subject of numerous investigations on cell permeability. Mostly a simple qualitative method is used (plasmolysis, staining of the sap, etc.), and several fundamental results have been attained in this way. The protoplasm seems to behave like a filter for molecules ("molecular sieve") and the rate of permeability is frequently a function of the diameter of the molecules. The absorption of electrolytes is a subject that has been less intimately studied by the champions of cell physiology, and a general weakness of the cell-permeability methods is the fact that only high concentrations are used, whereas plants under natural conditions absorb salts from very dilute solutions.

A thorough study of mineral nutrition implies chemical analysis of ions absorbed by the plant or thrown out in the solution (ion absorption and ion exchange). All chemical methods, even very sensitive microchemical ones, as for example spectral analysis, have a lower limit of sensitiveness. A direct determination of the absorption of salts by single cells is limited to the very large cells of *Nitella, Chara,* and *Valonia.* Hitherto but few experiments have been made

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with those cells with the object of elucidating the quantitative absorption of ions.

From the analytical point of view higher plants are much more favourable subjects for affording an exact knowledge of salt intake and exchange than cells of algae. Seedlings of cereals, peas, etc., are easily cultivated from genetically pure lines, and they can also, with but slight difficulty, be kept free from infections [Weissflog (53)]. The material is always ready for investigation and one can work under controlled conditions. In green algae carbohydrate assimilation sometimes interferes with the CO_2 balance and eliminates the interaction of bicarbonate ions in the absorption of anions. The non-chlorophyllous roots of higher plants provide more favourable conditions in this respect.

There seems to be no essential difference between cells and organs in respect of salt uptake. One can, for instance, regard a root tip as a large cell but with the difference that the total quantity of protoplasm is much larger in the latter. An absorbed substance has, in the single cell, to pass over a very thin layer of protoplasm whilst in the root it must traverse hundreds of protoplasmic layers in moving a few millimeters. It is therefore to be expected that the circumstances that determine differences in the movement of the ions will be considerably magnified in the multicellular organs in comparison to the single cells.

In experiments regarding the mechanism of salt uptake it is important that accumulation of salts as insoluble compounds in the plant should not interfere with the process of salt absorption. Plants accumulating calcium, for example, are less to be recommended. For the study of the fundamental laws of ion absorption and ion exchange a short exposure ought to be chosen. In this case the pure physico-chemical factors governing the ion movements are predominant. Suitable subjects are young seedlings of cereals cultivated in small vessels [fifteen plants in 50–100 cc. of solution (cf. 25, p. 11)]. The absorption and exchange of ions is then determined by analysis of the solution at the end of the experiment, due correction being made for loss of water by transpiration. Other subjects for similar experiments are slices of potatoes, carrots, etc., which are preferred especially by English and Australian investigators [Steward (48); Petrie (34)]. As Lundegårdh (25) pointed out, the reaction of plants toward electrolytes is much the same in experiments with concentrated and dilute solutions (plasmolytic or chemical methods),

but very important aspects of salt absorption, e.g. ion exchange, H—OH balance, ion antagonism, etc., afford quantitative methods of investigation.

For the conception of salt absorption as a manifestation of the interaction of ions with the colloids of the protoplasm the researches of Petrie (34), Brauner (4), and Pirschle & Mengdahl (38) have opened up new aspects. Lundegårdh (25) assembled results from extensive experiments with water cultures, pot cultures in quartz sand, and field experiments, and tried to give a survey of the importance of hydration, charge and size of the ions, and of interaction with colloids for the uptake of salts, and their distribution over the different parts of the plant. Due attention was paid to the solubility of salts of calcium and manganese and of phosphates in relation to the pH of the solution and of the influence of solid colloids such as humus and silica-gel, in the substrate.

The hydration is inversely related to the diameter of the ions: sodium ions, for instance, are more hydrated than potassium ions, and magnesium ions more than calcium ions. The hydration increases, furthermore, with the charge: the bivalent ions are more hydrated than the monovalent. The hydration is to be compared with a larger gross diameter of the ions and thus renders the uptake and translocation of ions in the colloidal meshwork of cells and tissues more difficult. This is one of the reasons why ions, and especially bivalent and trivalent ions, enter the protoplasm much more slowly than non-electrolytes of the same molecular size, and why, for instance, potassium ions generally move faster than sodium ions.

Another manifestation of chemical and physical properties of the ions is to be found in the absorption of colloids. The research work on soil colloids by Wiegner, Odén, and others has opened up new aspects as to the behaviour of organic colloids toward electrolytes. The ions are supposed to form shells or swarms around the colloidal particles. The attraction of the ions is influenced by their degree of hydration: the more hydrated ions are less intimately absorbed and are easily replaced by less hydrated ions. As a consequence of this fact, potassium, for instance, replaces calcium, the heavy alkali ions replace the lighter ones, etc. The protoplasm behaves quite similarly.

If roots of wheat seedlings are placed in a pure dilute ($M/400$) solution of CsCl, sodium and calcium are expelled into the solution. Potassium also replaces part of the Na and Ca in the root, but only to about ⅓ of the amount replaced by Cs [Lundegårdh, Burström,

Rennerfelt (28)]. In a solution of $BaCl_2$, five to ten times as much Na was expelled as in an equivalent solution of $CaCl_2$.

In consequence of their absorption quality heavy ions affect the swelling and permeability of protoplasm, determined by the endosmosis of glucose (cf. 28). In spite of their lower hydration the heavy cations in the Li–Cs and Ca–Ba series are therefore taken up to a lesser extent than, for instance, K and Ca.

The ion antagonism can also be regarded as a colloidal phenomenon. Earlier investigators have not clearly distinguished between the physiological effect of antagonism, growth and health of the plants, and the absorption of ions. It seems logical to restrict the conception of antagonism to the latter phenomenon and look upon growth, etc., as a consequence. Lundegårdh (25) made a study of the antagonism both in solutions and in pot cultures in quartz sand. The antagonism between K and Ca is very prominent, but there is also antagonism between Ca and Mg and between Na and K. The anions have also an influence upon the absorption of cations—K-ions are absorbed in descending order in the series $KNO_3 > KCl > KH_2PO_4 > K_2SO_4$ —but this is an effect of the energy balance in the system, protoplasm: electrolytes, where the absorption of ion pairs is preferred. The experimental results of ion antagonism, checked by extensive chemical determinations, are in conformity with earlier accounts by a number of authors (cf. 25, pp. 70–121).

Recent experiments by Kaho (19) and Gessner (12) show the interference between anions; the former demonstrated the poisonous effects of some metal ions, the latter made observations on salt absorption by submerged plants.

An important factor in respect of the action of anions is of course the secondary cH-regulations which occur in natural media and which control the solubility of phosphates, carbonates, manganese compounds, etc.

The antagonistic effect of cations on the structure of protoplasm has been studied ultramicroscopically by Hansteen-Cranner (14).

Recently, Sakamura (41) described some observations on the action of K and Ca on the consistency of protoplasm.

Yamaha & Ishii (55*a*) mention experiments on the influence of ions upon the coagulation of chromosomes in *Tradescantia reflexa*.

The ion antagonism seems to be a special case of ion absorption and ion exchange in colloids. The remarkable antagonistic effect of K toward Ca is analogous with the exchange of K and Ca in soil

colloids; K is attracted more by the negatively charged colloidal particles than Ca (54*a*). In the tissues the antagonistic effect of K increases as a consequence of its more rapid movement in the protoplasm. With the same external concentration K accumulates in the plant much more than Ca and the internal antagonism K : Ca is therefore prominent. The degree of antagonism varies of course with the ratio of concentrations of the two ions. The harmful effects of pure or unbalanced solutions may be due to the fact that ions are exchanged in a degree that disturbs the normal colloidal structure of the protoplasm.

Hydration and charge of the ions seem to determine the absorption of anions also. The anions NO_3, Cl, SO_4 were absorbed from equivalent solutions by wheat seedlings in the proportion 7.4:4.2:1 respectively [Lundegårdh & Burström (26)].

In accord with the importance of colloidal phenomena for salt absorption is the fact that the presence of solid colloids in the substrate restrains the absorption of Mg, Ca, and Mn, in comparison to K and Na, by the roots. It seems to be a competition between plant colloids and soil colloids, or it may be that the movement of the ions of higher charge is retarded in the colloidal medium, a fact that undoubtedly is of ecological interest. A suspension of colloids in the nutrient solution does not seem to have this effect [Niklewski (31)].

Respiration and absorption of ions.—Anions and cations are seldom absorbed in equivalent quantities from a single salt. The chemico-physical properties of the ions determine the absorption rate. As the sum of the cations is equal to the sum of the anions in the solution, the absorption is mostly associated with a displacement in the H-OH equilibrium. This means either an exchange of salt ions against H- or OH-ions in the protoplasm or the entrance of molecules [MOH, HA; cf. Osterhout (32)] of the acid and the base and a chemical reaction of these with the protoplasm. The former interpretation seems to be the simplest one as a logical consequence of the theory developed on the mechanism of salt absorption as a special case of ion exchange in the colloids of the protoplasm.

In the uptake of anions, bicarbonate ions and hydroxyl ions play a dominant rôle. Bicarbonate ions are unceasingly formed by the respiration. Lundegårdh & Burström (26, 27) showed that a rise in the CO_2 pressure in the solution increased the unequal absorption of anions by wheat roots. Thus the aëration of the substrate is a factor

in the regulation of the absorption of anions. With intensive aëration of a solution of a salt with slow-moving cation, for instance Na or Ca, and rapidly moving anion (NO_3) the pH will rise considerably. One-sided absorption of cations, for instance from KCl or K_2SO_4, is accompanied by low pH-values in the solution. The cells do not develop any buffer action corresponding to CO_2 production on the acid side, so that the pH value can fall below the toxic level. In nutrient solutions where several cations and anions are combined an equalization goes on between them, a well-known fact that cannot be neglected in the preparation of balanced solutions.

The respiration of the roots is always several times larger than the absorption of anions [Pirschle (37); Lundegårdh & Burström (26, 27); Åslander (2)], but there exists, nevertheless, a quantitative relation between them, as Steward (48), Lundegårdh & Burström (26), and Petrie (34) have demonstrated. Some of the investigations of Steward have already been mentioned in this *Review* [Hoagland (15)]. Steward used slices of potatoes, and the study was restricted to the absorption of one salt (KBr). Lundegårdh & Burström (26, 27) used young wheat seedlings, which were exposed by means of a special apparatus to constant aëration in 80 cc. of dilute salt solution. Numerous series of experiments were made with salts of the cations Li, Na, K, Rb, Cs, Mg, Ca, Sr, Ba, and the anions NO_3, Cl, SO_4 in $M/400$–$M/800$ solutions. In all the experiments the total balance of ions—absorbed and exchanged anions and cations, besides H^+, OH^-, and HCO_3^-—and the amount of respiration of the roots were quantitatively determined.

The total respiration R_t is the resultant of two parallel processes, called R_g and $k \cdot A$, of which the first is fundamental and appears in all cells, and the second is a multiple (k) of the anion absorption (A). $R_t = R_g + k \cdot A$. The lighter cations had, during the short time of the experiment (20 hours), no distinct influence on the respiration. k is characteristic of the nature of the anion (for NO_3, $k = 2$; for Cl, 3; for SO_4, 12). For the absorption of one ion of SO_4, 12 molecules of CO_2 are produced; for the absorption of one ion of NO_3 only 2 molecules of CO_2 are produced. There seems to be an inverse relation between these figures and the values for the mobility of the anions in the living substance.

The mechanism of salt absorption.—The unequal absorption of ions and the relation between respiration and anion absorption indicate that anions and cations are attracted to different parts of the

protoplasm. Some years ago Höber & Hoffmann (17) and Brooks (6) advanced the hypothesis that the protoplasmic membrane is built up of small patches with different transmitting power for ions. Some patches are permeable for anions, others for cations. This hypothesis has been criticized by Briggs (5) and others. It seems to be but slightly probable that permeability alone is the main factor in salt absorption, because permeability does not explain why, from an electrolyte, the cation is more absorbed than the anion or vice versa. The protoplasm must take an active part in the absorption, whereas in the uptake of non-electrolytes diffusion and permeability might be the main factors.

The protoplasm as a colloid contains charged particles and amphoteric ions (*Zwitter-Ionen*), which are surrounded by swarms of H- or OH-ions, and at the interphase between the nutrient solution and protoplasm an exchange with the ions of the electrolyte seems to occur. The proportion of negatively or positively charged swarms of ions that invade the protoplasmic surface depends upon the H-OH balance and the isoelectric points of the protoplasm colloids. The isoelectric point of the protoplasm seems to lie more or less on the acid side [cf. Pfeiffer (35), Strugger (49), Yamaha (55)]. The absorption of salts by the protoplasm has, according to this theory, the character of absorption reactions and the energy factor is the absorption potential and exchange potential of the charged colloidal particles or amphoteric ions. This theory is in accord with recent results on salt absorption already mentioned as well as with the work of Pantanelli (33), Pirschle & Mengdehl (38), and others.

The main difficulty in the problem of salt accumulation is not just the binding of salts in the protoplasm, but the fact that salts in inorganic form are stored up in the cell sap. This means that the attraction between the charged particles of the protoplasm and the salt ions is eliminated somewhere in the cell, which postulates energy supply. The production of organic acids and CO_2 and the existence of a constant difference of pH between the medium and the cell sap are of course factors that to a certain extent promote accumulation or one-sided absorption [cf. Osterhout (32); Lundegårdh & Burström (26), p. 245], but a theory for accumulation of general application cannot be built upon these facts. The researches of Steward (48) and Lundegårdh & Burström (26, 27) indicate that respiration supplies energy to some phase of salt accumulation. It seems probable that the protoplasmic layer has polarity—compare the statement [Damon

(8)] as to the different behaviour of outer and inner protoplasmic membranes—and that the oxygen attacks the assumed junction between protoplasm and anions (see *Respiration and absorption of anions*). The liberated anions will then combine with the absorbed cations and go into the cell sap as inorganic salt.

Distribution of salts in the plant.—As anions and cations are partly separated during absorption by the roots, they are also frequently distributed unequally in the plant. Some years ago Rippel (1926) published interesting observations on this phenomenon. Lundegårdh (25) gives numerous tables on the distribution of metallic cations (Na, K, Mg, Ca, Mn) in the roots, straw, leaves, and seeds of cereals, especially oats. Thoday & Evans (50) have published two papers on the distribution of certain anions and cations in the tissues of *Kleinia articulata* and other plants.

Nutrient and poisonous effects of some elements.—A number of investigations during the last decade have proved that several elements, hitherto believed to be dispensable, in reality form essential parts of the mineral nutrition. This is the case with boron, manganese, probably copper, aluminum, and so on. The chemical side of the function of these metals, mostly absorbed in very small quantities, is not yet satisfactorily elucidated.

Warington (52) has continued her research on boron and finds that the length of the day influences the appearance of the symptoms of boron deficiency.

The importance of manganese for normal growth of oats and other plants has been studied by Samuel & Piper (43), and Lundegårdh (25). The last-named showed that the hydrogen ion controlled the absorption of Mn from the soil. This is in agreement with the opinion of Piper (36) that the solubility of manganese in the soil is controlled by the soil reaction and by the oxidation-reduction equilibrium. A method of determination of the available manganese in the soil has been proposed by Steenbjerg (47).

The appearance of aluminum in plants has been thoroughly studied by Yoshiji & Jimbo (56). They used a microchemical method (Alizarin-S) and examined 674 phanerogams and 134 cryptogams of different families. Only certain plants showed high aluminum content, for instance, members of the families *Symplocaceae, Theaceae,* and *Lycopodiaceae.* An inhibitory action of Al-ions, partly in interaction with H-ions (see below) is postulated by Åslander (2*a*), Trénel & Frey (50*a*), and others.

Brenchley (4*a*) studied the action of copper, vanadium, and lithium on the growth of barley, maize, and peas. No definite case of stimulation was noticed. Vanadium had a decidedly toxic effect. Prát, Babicka & Políková (39) investigated the uptake of lead and thallium in the roots of *Zea mais* and *Vicia faba* and described pathological results on the structure. Sakamura & Yoshimura (42) believe that certain injurious effects on *Aspergillus oryzae* that are generally attributed to cH-relations are caused by impurities from Cu, Zn, and other heavy metals. Among reports on the stimulating effect of metals, we mention a paper by Birch-Hirschfeld (3). According to him molybdenum increases the nitrogen fixation of *Azotobacter.*

Burström (7) studied the poisonous action, or the stimulation, of a large number of inorganic compounds in the germination of *Tilletia* spores. The poisonous effect varies in a characteristic manner in the homologous series of elements. In the alkali series the poisonous effect decreases with increasing atomic weight. Ca is more poisonous too than Sr and Ba. The series Cr-Mo-U behaves similarly. Uranium has even a stimulating effect. The series mentioned lies on the positive side of the vertical columns in the periodic system. In the negative series Cu-Ag-Au, Be-Mg-Zn-Cd-Hg, B-Al-Tl, and Cl-Br-I the poisonous effect is the reverse; here it increases with increasing atomic weight. The atomic volume seems to be of importance, since elements with minimum atomic volume in the horizontal rows of the periodic system, viz. P, Ni, Co, Cu, Ag, Ce, Au, and Hg, all show a maximum of toxicity. For a discussion of the general physiological importance of the elements in dependence on the physical and chemical properties of the atoms and ions, see (25).

Among the common elements calcium is not indispensable for certain lower plants, such as *Azotobacter, Aspergillus* and other fungi, but it influences the cell division of *Micrasterias* according to Warén (51). Warén demonstrated an antagonistic effect of calcium and manganese. Sr can to a certain degree do service as a substitute for Ca; less favourable in this respect is Ba. The importance of calcium for the growth of different micro-organisms was studied by Rippel & Stoess (40). Ca compensates for the poisonous action of Mg. Sr has a similar effect.

As regards the effects of the less common alkali metals, the experiments of Alten & Gottwick (1), and Scharrer & Schropp (44) should be mentioned. The growth of oats was favourably influenced by lithium, whereas peas and wheat were injured. Maize was stimulated

by small amounts of rubidium. These results were obtained in complete nutrient solutions. In the absence of potassium a detrimental effect was always observed, showing that K cannot be replaced by other alkali metals.

There are a large number of papers that deal with agricultural science and the availability and effect on yield of the nutrient elements. Only a few of these papers need be mentioned here. Lemmermann, Jessen & Lesch (24) performed fertilization experiments with magnesium salts and on acid soils found a positive effect. Hoagland & Martin (16) investigated the absorption of potassium by barley and tomato plants with reference to the forms of potassium contained in the soil. Of special interest was the fact that no consistent relation between the actual concentration in the soil solution and the amount of potassium absorbed by crops was found. This is due to the fact that part of the potassium is replaceable, another part non-replaceable. The first part is more easily absorbed by the roots. There would perhaps have been a closer relation between the total amount of soluble potassium in the soil and the absorption.

Absorption of nitrogen and phosphate.—Stahl & Shive (46) worked with solutions in studying the absorption of nitrate and ammonia by oats and buckwheat. The solution had approximately the same proportion of nitrogen as NH_4 and NO_3. With oats the absorption of nitrogen showed partly a reverse course as compared with NH_4 and NO_3; it had with the former a maximum during the earliest period and with the latter a minimum. Also with buckwheat similar differences occurred, but this plant prefers NH_4 as nitrogen food, whereas oats prefer NO_3.

In their study of phosphate metabolism Weissflog & Mengdehl (53*a*) used plants in sterile culture [cf. also Greene (13)]. They examined the uptake of a number of inorganic and organic phosphorous compounds. Only salts derived from P_2O_5 proved to be suitable phosphorous sources. $MgNH_4PO_4$ had the best effect on maize. Phosphite and hypophosphite were not assimilated but showed, on the other hand, no detrimental effect. Pyrophosphate and metaphosphate were transferred into orthophosphate even in sterile culture. Phosphoric acid occurs in the plant chiefly as orthophosphate and seems to be translocated and stored up in this form, whereas the content of soluble organic bound phosphorous is negligible. Soluble organic phosphorous compounds such as glycerinephosphoric acid, hexosemonophosphoric acid, hexosediphosphoric acid, sucrosephosphoric acid, phytinic

acid, and nucleic acid may act as phosphorous sources for the plant. In some cases the result was even better than with inorganic phosphorous nutrients, which may partly be due to the buffer action. The esters are partly hydrolyzed so there is always a considerable amount of orthophosphate in the plant.

Of special interest is the sensitivity of rice to high concentrations of phosphate. Kimura (21) presumes that phosphoric acid is absorbed very rapidly by rice. In combination with Fe, insoluble iron phosphate is formed in the tissues. The detrimental effect of high phosphate concentration in the solution is compensated by soluble Fe salts, but as a consequence chlorosis can develop. The chlorosis increases with the Ca content of the solution.

Mineral nutrition of halophytes.—The nutrition of halophytes shows certain differences from that of other plants. They are exposed to very high concentrations of electrolyte. The earliest conceptions of the physiology of halophytes, that by means of transpiration regulations they limit their salt uptake, are wrong. Halophytes transpire as actively as mesophytes and absorb large quantities of salts. Characteristic is the large uptake of NaCl without detrimental effects upon the metabolism, whilst the cells of mesophytes are rather sensitive to high NaCl concentrations. The problem of halophytes is similar to that dealt with in the researches on the sensitivity of rice to high concentrations of P_2O_5, cited above. The protoplasm of every plant seems to be adapted to certain levels of ion concentrations. This seems to be one of the chief causes of the different geographical distribution of plants as far as edaphic factors are concerned. Iljin (18) studied the effects of Na, K, and Ca on the cells of halophytes and mesophytes. Cells of halophytes resist the action of pure NaCl in solutions in which cells of mesophytes die. On the contrary the former die in comparatively weak Ca solutions that are harmless to the latter. These interesting adaptations ought to be intimately studied from a physicochemical point of view.

Hydrogen-ion concentration and plant growth.—A characteristic feature of the recent studies of cH and its physiological effects is the consideration of the interaction of H-ions with the other ions in the solution. Of special interest is the antagonism, discovered ten years ago, between H-ions and Ca-ions, which is certainly of great importance for the growth of plants in acid soils rich in soluble nutrient salts. On the other hand more attention is now paid to the action of H-ions in the membrane equilibrium at the surface of the cells. Re-

cent studies of the isoelectric points of soil colloids and their importance for the chemical processes going on in the soil [cf. Mattson (29)] will be of interest for the clearing up of the problem of salt absorption, since there seems to be similarity between soil colloids and protoplasm in respect of electric behaviour.

The buffer action of the roots in the substrate is an important question. From what we have seen above, a considerable buffer action is exercised by the respiration. Buffer substances in the cell are, furthermore, phosphates and carbonates and organic acids with low dissociation in the cell sap. The buffer action of the cell sap of a few plants has been studied by Kozlowska (22). She found that there is a relation between the buffer effect and the ecological amplitude of the plant. Strong buffer action was shown by the saps of *Mercurialis perennis* and *Dentaria glandulosa,* whilst *Anemone nemorosa* had a weak buffer effect.

Of practical importance for water-culture experiments is to find nutrient solutions that have a constant or nearly constant pH. Zinzadzé (57) has proposed a number of such solutions in which he uses the buffer system $NH_4NO_3 + Ca_3(PO_4)_2$. As components of the solution on the acid side KCl is also used, and on the alkaline side, $KHCO_3$, and, at pH 7.5 to 8.0, $CaSO_4$ and $Mg_3(PO_4)_2$. In all solutions KNO_3, $MgSO_4$, and $Fe_2(SO_4)_3$ are present. The solutions cover the pH range, 4.9 to 8.0. Experiments with several plants showed a fairly good constancy of the pH through the whole vegetative period.

The question, frequently advanced, whether plants are capable of changing the pH value of the soil was investigated by Lemmermann, Fresenius & Gerdum (23). The "physiological reaction" of the fertilizer is of course important. The authors observed a slight displacement in the reaction of the soil under the influence of the crop, but it proceeded only slowly even in the case when the chalk was washed out to a considerable extent. Lemmermann indorses the view mentioned above that the content of soluble salts in the soil is of equal or greater importance to the plants than the actual pH.

The physiological action of the H-ions in the soil is complicated by the fact that the solubilities of several nutrients, such as Ca, Mg, Mn, Fe, Al, and PO_4, are to a large extent controlled by the pH-value [cf. Gaarder (9)]. The detrimental effect of high H^+-concentrations are frequently said to be due to the concentration of free Al^{+++} which increases with decreasing pH. Wieler (54) has recently studied the

development of sick soil in industrial districts. He regularly found in them sulphuric acid, aluminum, and iron, and a low pH. Wieler claims that the toxicity of the soils is not caused exclusively by the H-ions but also by the Al- and Fe-ions.

Mineral nutrition and metabolism.—The nutrient salts participate, after their uptake by the plant, in different manners in the building up of the organic substances. Especially the anions NO_3, PO_4, SO_4 and the cation NH_4 are more or less rapidly used up in the formation of proteins, phosphatides, etc. The purely chemical importance of the metallic cations in the building up of organic substances is still less known. A certain exception is made for iron and magnesium which have known functions in respiration ferments and in chlorophyll. The alkalies and Ca and Mg together with anions, for instance PO_4, undoubtedly have an essential task in the maintenance of a suitable colloidal consistency in the protoplasm, for the turgescence of the cell, for buffering against pH changes, and as regulators in enzymatic processes. A distinct relation between the uptake of nutrient salts and the intensity of metabolic processes, such as carbohydrate assimilation and the formation of proteins, is therefore always to be found.

The relation between potassium nutrition and carbon-dioxide assimilation has been studied by Lundegårdh (25) and Gassner & Goeze (10, 11). The last-named authors found in young leaves of wheat an increase in the assimilation rate with potassium deficiency, but Lundegårdh (25), who used leaves of oats, did not find any distinct difference in assimilation by leaves of different potassium content (minimum, 0.64 per cent of K in the dry substance with total potassium deficiency).

With nitrogen deficiency the leaves showed a distinct increase in the assimilation intensity in comparison to plants that were given a medium NO_3 supply. Müller (30), on the contrary, states that leaves of *Sinapis alba* show a lower assimilation rate with nitrogen deficiency.

The earlier investigations on the relation between mineral nutrition and carbon-dioxide assimilation give somewhat contradictory results also. This is hardly surprising, for different plants and leaves of different ages were used. It is also important that the potassium content of the plants used should be controlled, as even in plants grown without any supply of potassium the leaves contain this element (see above). A direct chemical relation exists between external conditions and CO_2 assimilation in respect of CO_2-pressure, light,

and temperature, but *a priori* in the indirect relation between mineral nutrition and assimilation very fluctuating results are to be expected.

As regards the influence of potassium nutrition on protein metabolism, Schmalfuss (45) did not find any close relation. In agreement with other investigators he states that with potassium deficiency the water content of the fresh plant is low and the nitrogen content high.

A starting point for a future analysis of the relation between mineral nutrition and metabolism and growth should be the mobility of the nutrient substances. These move, certainly, as compounds of comparatively low molecular weight and partly, in the case of phosphates and nitrates, as inorganic ion pairs. It seems probable that the readily moving cations play a rôle as partners in the translocation of nutrient anions.

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METABOLISM OF CARBOHYDRATES AND ORGANIC ACIDS IN PLANTS (EXCLUSIVE OF BACTERIA AND FUNGI)*

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Carbohydrates

Methods for determination of carbohydrates in plants.—The difficulties in the chemical analysis of plant materials lie less in the often small amount of the carbohydrates than in the difficulties of isolation and of eliminating the interfering action of accompanying substances, to which especially enzymes and non-carbohydrate reducing substances belong. As Schroeder & Hermann (45) have again been able to show, it is necessary to give special attention even to the preparation of the plant material to be analyzed. The use of expressed juice for the determination of the several carbohydrate fractions is to be rejected on account of the postmortem enzymatic changes which set in extremely rapidly, unless care is taken to kill the protoplasm and simultaneously inactivate the enzymes. Dry heat also yields inexact results, especially when prolonged at a low temperature [e.g., 44 hours at 50° C., Archbold (2)]. On the other hand, treatment with steam or boiling alcohol is of value. Similar observations were made by Morris & Wesp (33) and by Sayre & Morris (41), who ascertained that the process which has been often recommended, of freezing the material before grinding and expressing its sap, is not sufficiently rapid to prevent, for example, the hydrolysis of sucrose. However, according to Phillis & Mason (37), if definite conditions are maintained, the freezing method yields useful results, and is even superior to the method of extracting with alcohol or with water.

The clarification of the plant-sap or extract and its freeing from other reducing substances has long been carried out especially with neutral or basic lead acetate, the lead being removed with Na_2HPO_4 or sodium oxalate. Under certain conditions, it is recommended that this method of clarification be modified: in the presence of much protein but absence of amino acids and other reducing electrolytes Munday & Seibert (34) employ Folin-Wu's tungstate reagent; but if free amino acids and other nitrogen-containing electrolytes are

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present, it is recommended that the hydrolysis be followed by a precipitation with mercuric sulphate. Certainly it is not possible to accomplish in this way the complete removal of the reducing non-sugar substances. In working with blood and urine, the method of yeast fermentation for sugar determination has been employed with increasing frequency. In the case of plant material this method is even less dispensable. Kerstan (25) has utilized it extensively, and through this, as well as through the application of a system of charcoal adsorptions and elutions, he has made material progress in the specific determination of particular carbohydrate fractions, aimed at already by Lehman (31).

Phillips (38) has compared the methods of determination which depend upon reduction and has shown that they are not equally reliable for different plant materials. He therefore believes that the material from a given plant must be tested by different methods, and that the method which gives the lowest reducing values should be regarded as the most suitable. Thus he assumes that the higher values obtained by the other methods are due to non-sugar substances to which these reagents are sensitive. For that reason, the particularly sensitive Hagedorn-Jensen method requires a very careful elimination of reducing non-sugars, and the less sensitive methods of Folin (alkaline copper tartrate) and of Shaffer-Hartmann are often used [e.g., by Schlenker (43)].

Individual carbohydrate fractions are usually determined by difference. Phillis & Mason (37) first determine the initial reduction, then oxidize the glucose with alkaline iodine solution and obtain the fructose by difference. Sucrose is determined by means of the measured increase in reducing value after inversion with invertase. For the determination of the total reducing value, Dastur & Samant (14) use a colorimetric modification of the Folin-Wu method (by applying phosphomolybdic acid).

After removal of the soluble sugars, Shriner (48) determines starch by hydrolysis, with takadiastase, into glucose and maltose. After the combined reducing power of these two has been ascertained by means of the Shaffer-Hartmann modification of the Munson-Walker method, the amount of starch is computed by a simple calculation from the determined values.

An iodine-colorimetric method for the determination of cellulose, which has the advantage of speed and yields exact results, has been suggested by Paloheimo & Valavaara (36). The method requires that

the directions be observed with painstaking exactness and that the cellulose content of the plant material to be examined be approximately known.

No less are the difficulties which are encountered in the analytical determination of glucosides. At once, it must be emphasized that any method makes possible only the determination of sugars of glucosidic linkage, but that the further progress in the physiological research of metabolism depends, to a large degree, upon whether or not one succeeds in including the "aglucones" (non-sugar residues) in the quantitative analysis. Klein & Linser (26, 27) were able to carry out quantitatively satisfactory fluorometric determinations of aesculine in *Aesculus hippocastanum* with Kleinmann's nephelometer, and Kerstan (25) succeeded in showing that a system of charcoal adsorptions and elutions developed by him makes possible the determination of several phenolglucosides (aesculine, arbutine, and salicine) in a mixture of the common carbohydrates. These procedures are more reliable and exact than the methods depending on a combination of polarimetric and reduction determinations, developed by Bourquelot and his students, which are used by French investigators and have, in fact, been of good service in the discovery of new glucosides. In so far as the aglucones can be eliminated through adsorption by charcoal, the method of Kerstan is to be preferred to that of Bourquelot, especially in view of its more extensive range of application, since Bourquelot's "enzymolytic reduction coefficient" is applicable only when the aglucone does not itself reduce.

To the considerable difficulties encountered in the analytical determination of the several carbohydrate fractions, there is to be added, as in other fields of the physiology of metabolism, the fact that the usability of the analytical data depends also, fundamentally, on the correct choice of a basis of measurement. If the latter does not remain constant within a certain plus or minus value, large errors often result, not infrequently leading to conclusions diametrically opposed to the true state of affairs. For that reason Denny (16) properly investigates carefully the unit of measurement most suited to an exact determination of the diurnal variations in the content of the leaves. He finds that neither fresh weight nor leaf area is reliable, for both are variable as a consequence of the fluctuating water content. If opposite leaves or leaflets be cut off, the one at the beginning of an experiment, the other at its end, and both be examined with regard to the total content of all constituents, the error may be avoided. The same holds

for half-leaves. Also the "residual-dry-weight" method, in which all constituents are computed on a dry-weight basis, after subtraction of the total carbohydrates, is often reliable, for the carbohydrates show the greatest diurnal variation. In many plants, more than half of the carbohydrate content accumulated at the end of the day disappears during the night.

The error due to an unsuitable basis of measurement pertains also to the investigations which Kurssanow (30) conducted on fruits of *Eriobotrya japonica*. He uses fresh weight as a basis, although this is just as unsuitable as any other basis, for in growing organs all bases change together. The use of fresh weight as a basis in the case of the ripening of seeds is especially erroneous on account of the continuous reduction of the water content. For growing organs only data on the total content of every substance are of value.

Occurrence of particular carbohydrates.—Trehalose found in a fresh-water red alga (*Lemanea nodosa* Kütz) by Colin & Augier (11) is to be mentioned among the newly discovered simple carbohydrates. In confirmation of previous statements, Colin & Belval (12) found raffinose in different cereals, in which it is localized in the grains with the exception of the integuments and the aleurone layer. It also occurs together with sucrose in the seedlings (*germes*).

Sugar alcohols.—These have recently become known as typical metabolic products of certain plants, particularly the lower plants (*Phaeophyceae*). Haas & Hill (22–23) established the occurrence of sorbitol and dulcitol in a *Rhodophyceae* (*Bostrychia scorpioides*), limited to this species. Mannitol, which generally occurs in the *Phaeophyceae*, was also absent in the other *Rhodophyceae* examined. Haas & Hill are inclined to consider these sugar alcohols as secondary products derived from sugars. The absence or scarcity of free sugars in the *Fucoideae* could be conditioned by the rate of formation and the rate of utilization being but little different. These are, of course, mere assumptions which, however, illustrate how far we still are from a causal understanding of this anomalously regulated carbohydrate metabolism.

It becomes increasingly clear that sugar alcohols are also fairly commonly distributed in the higher plants. Obaton (35) found dulcitol as well as sucrose in the leaves of *Evonymus europaeus*. He assumes that dulcitol as well as mannitol, which he has found in *Apium graveolens*, are products of chlorophyll assimilation. Asai (3) disagrees with him, since he fails to observe any correlation between the

conditions of assimilation and those of mannitol formation in the different mannitol-containing plants investigated. In these plants, mannitol is found in all the organs of vegetation, always more abundantly in leaves and bark than in the woody or root tissues. The mannitol content of the leaves increases in the autumn and reaches a maximum in about February (up to 12 per cent of the dry weight in *Veronica Tourneforti*); it then rapidly decreases and eventually completely disappears in May. This seasonal trend was found to run in a similar direction to that of the reducing sugars and in the opposite to that of starch.

Polysaccharides.—Among the polysaccharides, many plant mucilages and reserve materials are being investigated at present with regard to their chemical constitution. With this foundation, the study of the rôle of these substances in plant metabolism may later be approached. Although simple hexosans, pentosans, and inorganic-acid esters of these compounds occur in plants, in most of the investigated cases polysaccharide-uronic-acid complexes are concerned, a large number of which have been isolated and determined.

The uronic acids identified are almost exclusively galacturonic and glucuronic acids. Schoeffel & Link (44) verified the presence of mannuronic acid in *Macrocystis pyrifera* as a component of alginic acid, which may be connected with the occurrence of mannitol in this plant. Renfrew & Cretcher (39) have found that in the mucilage of the quince seed the complex consists of the following components: arabinose, a mixture of methylated and unmethylated aldobionic acids (among which xylose-hexuronic acid was found), and a cellulosic fraction. This unexpected appearance of cellulose as a component of mucilage was also confirmed by Bailey & Norris (4) in the case of seeds of *Brassica alba* (50 per cent cellulose) and linseed (small amounts of cellulose). The rest seems to consist of a rhamnose and a galactose-galacturonic acid, together with arabinose. Bailey & Norris attribute the formation of uronic acid from pre-existing hexosan material to the peroxidase system contained in sclerenchymatous palisade cells which are situated close to the mucilage-secreting cuticle cells. The fact deserves special mention that cellulose, otherwise insoluble, is very easily soluble in the presence of uronic acids. Gough (21) split, through hydrolysis, a gummy-like polysaccharide from the pollen of timothy grass into *l*-arabinose, galactose, and a non-reducing acid. Anderson & Kinsman (1) investigated a hemicellulose of cotton-seed hulls. This hemicellulose is composed of *d*-glucuronic acid, *l*-xylose,

and also presumably of a compound as yet unidentified. They believe that gummosis in plants can often be brought about by hydrolysis of the ester linkage, which most probably consists of a bond between the carboxyl group of the hemicellulose and the hydroxyl group of the cellulose. This hydrolysis is, presumably, also associated with other changes in the molecules. Sands & Gary (40), however, could not find any relation between the hemicelluloses and the gum of the mesquite wood, which would, perhaps, indicate that the hemicelluloses are precursors of the gum. The hemicelluloses of the mesquite wood consist of a mixture of two polyxylonomethoxy ureides.

Daoud (13) found in the seeds of *Trigonella Foenum-Graecum* a reserve polysaccharide which he identified as a silico-phosphoric ester of mannogalactan and which resembled amylopectin and lichenin. The analogy with starch is enhanced by the fact that this polysaccharide is apparently found in various forms and is probably esterified to different degrees, yielding compounds possessing various solubilities. During the germination of the seeds the mannogalactan is decomposed and its components undergo a steric transformation (the mechanism of which as yet is not understood) into glucose and fructose and, secondarily, into sucrose.

The physiological behavior of carbohydrates.—The work to be discussed below deals, without exception, with the changes in the carbohydrate content of plants brought about by the action of certain external factors and changing internal conditions, e.g. during development. Many carbohydrates are utilized or accumulated, or transformed into each other or a different substance, so that with the characteristic abundance in the plant of simple sugars, di- and poly-saccharides, there results a constantly changing and often complicated interplay. Since carbohydrate metabolism must be regarded as the central problem of plant metabolism, it is especially to be regretted that the application of our analytical methods to plant materials still involves considerable errors, which are mentioned in the introduction. Therefore only a limited value may be attached to much, if not most, of the work pertaining to this subject.

Internal factors.—Some work deals with the natural trend of carbohydrate changes at different times and under certain physiological conditions. Phillis & Mason (37) continued their work on carbohydrate transport in the cotton plant and sugar distribution in the leaf. Glucose, fructose, sucrose, and an unknown polyglucoside present in small amounts were definitely identified. Sugar migrates from the

tissues of assimilation to the phloem of the leaf veins and the stem, mostly in the form of sucrose. Belval (5) obtained different results in the case of the banana plant. He followed polarimetrically the changes in the carbohydrates in various separate plant organs. According to his view, sucrose is a predominating sugar in the lamina and is indicated as the first demonstrable assimilation product. During the migration through the middle vein and leaf petiole, inversion progresses so that a mixture containing very little sucrose migrates to the stem and the fruit, where first invert sugar, and then very slowly also sucrose, change to starch. During ripening, starch is transformed into invert sugar, passing through the intermediate stage of sucrose. Dextrins could not be detected anywhere. According to Franquet (18), also, these intermediate substances are entirely lacking in the reserve organs of various plants during the formation of starch from the incoming sugars. Even maltose could be found only in *Bolbostemma* where, however, its direct connection with starch formation remains questionable, as is also true in the case of stachyose in bean seeds. Since starch breakdown during germination in no instance led to the detection of dextrins and maltose, the formation and hydrolysis of starch must proceed very rapidly.

Another series of investigations deals with carbohydrate metabolism during ripening of fruits and seeds. Little value can be assigned to this work, partly on account of methodical errors (analytical, basis of calculation). Kurssanow (29), for example, presents his data on fruits of *Eriobotrya japonica* in percentage of fresh weights. It should be mentioned that during ripening, fructose, sucrose, and malic acid accumulate in the pericarp, while maltose (?), tartaric acid, and amygdalin disappear almost completely. Citric acid, dextrins (?), starch, hemicellulose, cellulose, etc., decrease in amount. In ripening seeds, an accumulation of starch, hemicellulose, cellulose, and amygdalin is reported.

Archbold (2) investigated very thoroughly changes in the content of many compounds during the ripening of apples. The method of determination of carbohydrates used takes into consideration many sources of errors, but unfortunately not adequately with respect to reducing non-sugars. Of her results, it should be mentioned that in one variety, the only product of starch hydrolysis seemed to be fructose, and that also during cold storage the same sugar accumulated as a result of sucrose inversion. From this it is concluded that glucose is transformed into fructose, which appears as an end product of sugar

transformation in aging tissues from which migration no longer takes place. On the other hand, with increasing sugar consumption, fructose is increasingly utilized. Widdowson (58) investigated the behavior of starch and hemicellulose in the same material. Starch appeared in young fruits by the middle of June and, after a maximum in summer, disappeared completely by the end of October. Two substances were extracted with hot water from the alcoholic residue, one a polyuronide, and the other a polysaccharide with no uronic groups. Upon hydrolysis, both yielded arabinose and therefore were presumably not utilized in respiration. These compounds were constantly accumulated and did not decrease even during storage. The same was also true for a substance extracted with *N*/75 HCl, which, in structure and function, was related to pectin.

Bridel & Bourdouil (8) studied the seeds of two varieties of peas, according to methods used in France, and they have taken into consideration the presence of stachyose. One of the two varieties (*"le délicieux"*), characterized by wrinkled green kernels, contained in the seeds more soluble carbohydrates and less starch. This was due to a smaller capacity for starch formation and not to an actual high sugar content. The ripe stage of this variety corresponded to a younger stage of the other variety (*"Très hâtif d'Arras"*), which had a smooth surface and was rich in starch.

External factors.—A series of investigations deals with the behavior of carbohydrates in plants in so far as it depends on external conditions. Stanescu (50) and his co-workers followed, over a period of many days, with the aid of the simple iodine test of Sachs, the diurnal changes in starch accumulation. They studied the leaves of twenty species and found the behavior to be different even among plants of the same species. These differences could not always be accounted for by external conditions. Gillot & Vigneron (20) investigated the seasonal variation of carbohydrates in the underground shoots of *Epilobium hirsutum* L. They report, without data on methods, the total content of starch, and reducing and hydrolyzable sugars, the latter expressed as sucrose. We note that, not the rhizome, but only the shoots from this (*drageons*) store large quantities of starch, especially from July to October. The starch is then utilized until spring. The work of other investigators is concerned with the effect of temperature. Schaffnit & Wilhelm (42) found an increase in the sugar content of the cell sap of potato and tomato plants at falling temperature, similar to that observed in winter cereals, particularly in

the case of nitrogen, potassium, and phosphorus deficiencies. It has been known for a long time that the temperature during germination influences materially the development of cereals in the later stages. But the susceptibility to *Fusarium* likewise depends to a large degree on soil temperatures prevailing during germination. Gäumann (19) attempted to determine whether or not this effect was brought about by a chemical change in the composition of the seedling as affected by soil temperature. In fact, marked changes occurred in relation to temperatures of germination, among others the carbohydrate content, including xylan, cellulose, and lignin, which are used in building the cell wall, and are important in connection with the penetration of parasites. The interesting conclusions of the author concerning this point should be especially emphasized. The effect of the hot water-bath treatment, commonly used to break the rest period, is due only partially to a temperature effect. Hasselbring (24) investigated the effect of this process on the carbohydrate changes during storage of narcissus bulbs. He found that this effect manifested itself in an acceleration of starch disappearance, and in an increase in sucrose and reducing sugar. The treated bulbs are thus at the time of planting in a more active internal condition, on account of their larger content of easily metabolized carbohydrates.

The problem as to the manner in which various mineral nutrients influence the carbohydrate content and that of other substances is of great practical importance. Thomas (53) conducted careful investigations on this question, using genetically similar young apple seedlings grown in the same soil. It is worth noting that nutrition in a nitrate-free medium decreased the concentration of the simple sugars, and the activity of invertase. The accumulation of starch is connected with cessation of active growth, and is even considered by Thomas as the cause of the checking of growth. The quantity of starch increases with the addition of definite mineral elements in the following order: N, P, K < N, K < N < P, K < P, and the disappearance of starch during growth is particularly rapid when nitrate is added.

The water content of plants also has a marked influence. Vassiljew observed an increase in soluble carbohydrates in wheat plants when water was deficient. On the other hand, with a more readily available water supply, a decrease in soluble carbohydrates was found, despite a continued or increased photosynthetic activity. In a recent contribution (54), the same writer deals with the influence of weather and finds that with the same objects, the more favorable the conditions

for transpiration, the higher is the sugar content. The fractions of different sugars vary with the weather factors. Thus, for example, insolation being the same, sucrose predominates in cool weather, while on warm days its amount is much smaller. The age of the leaves, contrary to earlier observations, is said to have no great importance for the content of carbohydrates.

McDougal, Richards & Spoehr (31*a*) are of the opinion that the basis for continued succulency is the transformation of hexose polysaccharides with low imbibition power into pentose polysaccharides with high hydration power. Wood (60) also finds that the carbohydrate metabolism of plants of arid regions is characterized by a high content of pentosans, and that the imbibition power of the pentosan-protein-colloids is responsible for the drought resistance of the thick-leaved (succulent-leaved) plants. While the succulent plants proper, form simple pentosan mucilage, which is soluble in the plasma of the water tissue, the hard-leaved (sclerophyllous) plants are, on the other hand, distinguished by a higher content of uronic acids and aromatic lignin linkages. Although this evidence may be convincing, yet the problem cannot be considered as solved. Thus, Evans (17) doubts whether the small content of water-soluble pentosans in *Kleinia articulata* is sufficient to change the constitution of the plant, and to provide it with massive storage tissue, since there are plants (e.g., Fuchsia) which ordinarily are not regarded as succulents, and yet contain more water-soluble pentosans than *Kleinia* does. The same relation holds true also, according to Evans, in regard to the pentosan fraction extractable with 1 per cent HCl. A considerable fraction of the pentosans is extractable only with 12 per cent hydrochloric acid; this fraction, whose chemical nature is as little known as that of the previously mentioned fractions, is presumably of little significance to the pentosan theory of succulency.

Glucosides

Our knowledge concerning the presence of glucosides in plants is constantly increasing, particularly in the case of those which are pharmacologically important. Unfortunately, we are, as yet, in the dark concerning their rôle in the metabolism of the plant. This problem requires a careful following of changes in the glucoside content of various plant organs during their natural development, as well as under special experimental conditions. This, however, is limited by analytical methods.

New glucosides were found recently in many plants. Charaux & Rabaté (9) found, in the bark of *Salix purpurea* L., a new glucoside designated as "isosalipurposide," along with salicoside and the colorless salipurposide (dehydrophlorhizide) discovered by them the previous year. The former imparts a golden yellow color to the inner bark, and it yields *d*-glucose and "salipurpol" with emulsin and dilute sulfuric acid. Salipurpol is presumably related to the flavonols. By careful elimination of enzymatic action during the preparation, Stoll & Kreis (51) succeeded in isolating from *Digitalis purpurea* a new glucoside instead of the well-known digitalin. This new glucoside, named by these investigators "purpurea-glucoside A," was found to be richer in sugar, and pharmacodynamically more active than digitalin. The leaf powder which contains "digipurpidase" splits each molecule of "purpurea-glucoside A" into one molecule of digitoxigenin, one molecule of glucose, and three molecules of digitoxose ($C_6H_{12}O_4$). Likewise from *Digitalis lanata,* to which recently much attention has been paid as being still richer in glucosides and more active, the investigators referred to above isolated three crystallographic isomers, namely, "lanata glucosides A, B, and C." Each of these three glucosides contains three molecules of digitoxose and one molecule of glucose, the latter being split off by the leaf enzyme "digilanidase."

Schmiedeberg in 1882 (43*a*) discovered two digitalis glucosides, neriine and oleandrine, in the leaves of *Nerium oleander.* According to Tanret (52), and contrary to the opinion of Schmiedeberg, these glucosides are markedly different from digitalis glucosides, as well as from strophanthines, to which Leulier (1912) related them. Neriine and oleandrine could be obtained only in the amorphous state.

Vintilesco & Ioanid (55) found three "vincosides" in *Vinca herbacea* L., *minor* L., and *major* L. These vincosides are hydrolyzed by emulsin. Two of these, designated by A, are precipitated by lead acetate, while the third, B, is not precipitated. The "enzymatic reduction-indices" are given. The glucoside content of the plant reaches a maximum in May and January. Sosa (49) discovered a new glucoside "betulosine" in the bark of *Betula alba* L. This compound is nitrogen-free, colorless, and is equally hydrolyzed by emulsin. The probable composition of its aglucone is $C_6H_{14}O_2$. Kramer (28) continued his studies on philyrine (called by him philyroside), found in the bark of twigs of *Philyrea latifolia* L. The empirical formula of philyrine is $C_{27}H_{24}O_{11}$. It is hydrolyzed by emulsin and by powder from *Aspergillus niger,* but not by dry bottom-yeast. The aglucone, philygenol,

possesses a free phenol and three methyl-ester groups, but lacks aldehyde, keto, and carboxyl groups, as well as double or triple bonds. In the same bark, Kramer also found a second glucoside, "syringine" (which he named "syringoside"). This glucoside is very widely distributed in the *Oleaceae*. It yields *d*-glucose with emulsin, its aglucone conforming to the formula $C_{11}H_{14}O_{11}$. It is claimed to contain two free hydroxyl groups, two free methoxy groups, and one ethylene double bond.

The number of investigations dealing with the physiological rôle of glucosides in plants is very small. Klein & Linser (*26, 27*) investigated the distribution and the metabolic fate of aesculin in the various organs of *Aesculus hippocastanum* L. Determinations were carried out fluorometrically, with the aid of the nephelometer of Kleimann, in the alcohol extracts of the dried material, during two vegetative periods. Unfortunately this method also fails to take into account the aglucone. The largest amount of glucoside is present in the bark and bud scales and increases with age. However, at least for a limited time during a period of pronounced need (for example during the unfolding of buds), the glucoside may serve as a reserve material. In the bark the aesculin content exhibits two seasonal maxima, one in winter until the beginning of spring, the other in the summer. In between are two minima, one at the beginning of the growing season and the other before defoliation. It is, as yet, undetermined whether or not the glucoside migrates to the leaves before defoliation or is consumed.

Braecke (*7*) arrives at similar conclusions in her investigation of the seasonal changes in various plants of the content of glucosides in relation to other carbohydrates. She considers aucuboside, taxicatoside, and piceoside as reserve materials. Meliatoside and gentiopicroside, on the other hand, exhibit a maximum at the beginning of the growing season.

Deleano & Andreesco (*15*) found that salicin accumulates in the leaves of *Salix fragilis* only during the period of growth. At the end of this period a maximum is reached, followed by a decrease.

At this point, it may be appropriate to mention the studies of Michel-Durand (*32*) on the physiology of the origin of tannic substances. An attempt was made to ascertain in which sphere of metabolism tannin in *Castanea vesca* and *Aesculus hippocastanum* originated, by comparing the fluctuations of the tannin with those of the carbohydrate and the nitrogen fractions. As was to be expected, cor-

relations were found only with the carbohydrate metabolism. But the chemical nature of this relation was not investigated.

Organic Acids

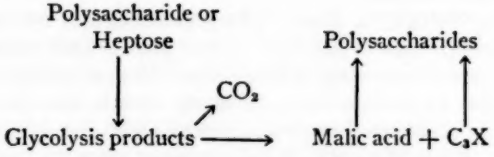
Our knowledge concerning the position of organic acids in plant metabolism remained for a long time unsatisfactory, particularly on account of analytical difficulties and deficiencies. Only in recent years has progress been made.

1. Thus Ruhland & Wetzel (*39a*) established, a few years ago, the physiological type of ammonia or acid plants. Such plants possess a low pH, and a very close relation exists between nitrogen metabolism and acid formation, inasmuch as the organic four-carbon acids (malic acid) are derived from mono-amino acids through deamination and ammonia liberation. It was recognized that the hydrogen-ion concentration acted as a limited factor in this process. From the physiological point of view, it is important that in this type of plants the organic acids neutralize the toxic action of NH_3; while in the case of plants having a higher pH this detoxication takes place through the formation of amides. Plants of the latter type are designated by Ruhland & Wetzel as "amide plants." The investigations of Kultzscher (31) confirm these conclusions and provide them with a more extensive basis. He shows, contrary to the views held by Löffler & Steiner (*50a*), that the mechanism of detoxication can be recognized only when the plant is compelled to detoxicate an excess of ammonia, as, for example, in the case of a strong protein decomposition.

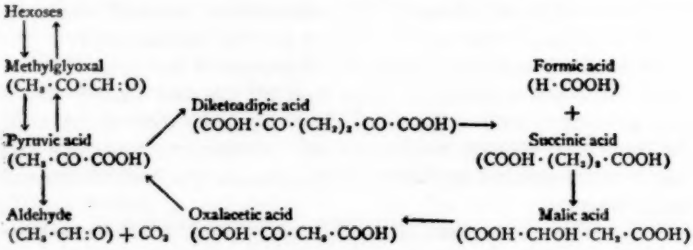
2. In the *Crassulaceae,* malic acid is generated in a quite different way. These plants possess a fairly high pH (greater than 5) and a large content of malic acid, which shows a regular diurnal variation. As Wolf (59) showed, a relation exists between acid formation and carbohydrate metabolism, but none between nitrogen metabolism and acid formation.

According to Bennet-Clark (6), sedoheptose, found in certain species of *Sedum,* is transformed during the night into malic acid through a "glycolytic process." The accumulation of malic acid (whose determination was unfortunately based only on titratable acidity) occurs at a high carbohydrate content of the tissues when the rate of malic-acid formation is faster than that of its further transformation to carbohydrates. Carbon dioxide may originate as an intermediate product of glycolysis but is not derived from malic acid. A fraction of the malic acid disappears on exposure of the leaves to

darkness and, according to Bennet-Clark, this fraction is transformed entirely to polysaccharides. A hydroxy aldehyde (possibly glycolaldehyde) is an intermediate product of this transformation. Further details concerning the chemical mechanism of this cycle are not given. Neither were investigations undertaken to establish a chemical balance in support of this view. The decrease in acidity on illumination was attributed to the high temperature of the illuminated plant organs.



According to Wetzel & Ruhland (56) and Wolf (59) the principal difference between the carbohydrate metabolism in the *Crassulaceae* and other plant types consists in the following: At moderate and low temperatures the three-carbon compounds, produced in the normal course of carbohydrate metabolism (Neuberg), are not further broken down but, instead, are synthesized by oxidation into malic acid according to the process postulated by Toeniessen & Brinkmann (53*a*) for the building in muscle of succinic acid from pyruvic acid.



The transformation of succinic acid to malic acid in the organism can also be readily visualized. The fate of formic acid is not as yet known. At any rate the above scheme satisfies the necessary requisites for oxidative synthesis of malic acid. As optically active compounds, generally, are selectively affected in metabolic processes, so in this instance the diurnal change in the acid takes place chiefly in the *l*-form of malic acid. It has been often observed that at the end of an intense

phase of deacidification, the *d*-form of the acid exceeds that of the *l*-form, as has also been shown by Bendrat (5*a*).

The checking of the decomposition of pyruvic acid is probably due to the checking of the activity of carboxylase by acetaldehyde, as Wetzel (57) found in the case of yeast. His conclusions are of importance in so far as they simplify and illuminate our conception of sugar metabolism. According to Wetzel, the regulation of sugar metabolism is largely a question of available hydrogen acceptors and donors. The nature of the donors is determined by the extent of activity of the ferments acting upon them. The process is predominatingly an interaction of oxido-reductase and carboxylase. If the carboxylase is inactivated, then the pyruvic acid produced as an intermediary product is either reduced to a hydroxy acid (lactic acid) as experiments of Neuberg & Kobel (34*a*) on the action of macerated tobacco leaves on zymophosphate make evident, or changed otherwise, perhaps to malic acid. If, on the other hand, carboxylase is active, the reduction would first take place at the decarboxylated pyruvic acid (acetaldehyde). In this way the assumption of a special keto-aldehyde-mutase (Neuberg) would seem superfluous. The fact that aldehydes in general check carboxylase activity, would explain why methyl glyoxal (as aldehyde) is transformed by zymase preparations into lactic acid, and not broken down into alcohol and CO_2.

Wetzel & Ruhland (56) found that the aldehyde content of leaves of *Crassulaceae* increases during the phase of malic-acid formation. It is obvious, however, that at the same time inhibition of oxidation by carbon dioxide occurs. Carbon-dioxide formation through splitting off of the carboxyl from pyruvic acid is inhibited more than oxidation. The accumulation of acetaldehyde, according to Ruhland & Wetzel, cannot be directly related to an inhibition of oxidation. Possibly there exists an indirect relation such that inhibition of oxidation, resulting in accumulation of acetaldehyde, brings about an increase in intramolecular respiration. The primary cause of aldehyde accumulation is probably due to some disturbances in the mechanism of aldehyde destruction.

In the case of *Crassulaceae* the inhibition of carboxylase activity falls off at higher temperatures, so that the pyruvic acid produced, both directly from carbohydrates and also from malic acid (presumably through oxalacetic acid) can be broken down. By this process, a better utilization of the respiratory materials is achieved. Indeed, analytical determinations have shown that at 35° C. less carbohydrate

is consumed than at 20° C. It is assumed that the products of primary sugar cleavage are resynthesized into carbohydrates by way of the Pasteur reaction. The acid metabolism of the *Crassulaceae,* thus conceived, is parallel to that of animal and yeast glycolysis.

Schwarze (46), in agreement with Wolf, found that in the case of deacidification by light, high temperature and secondary light influences are particularly important. The connection between assimilation and deacidification is explained by an increase of oxygen supply to the tissues, and in particular by the decrease in their CO_2 tension.

The reason for the similarity of carbohydrate metabolism of certain plants to that of the *Crassulaceae* type is not as yet clear. According to Schwarze, and partially in agreement with older works, the diurnal changes in metabolism of organic acids are not confined to succulents only. This observation, and the fact that the concentration of acids might be the same in succulents and non-succulents cannot be attributed to a succulent-like structure. Schwarze showed especially that the accumulation of organic acids is not caused by an inadequate penetration of oxygen into the tissues. Furthermore, the same writer disproved (in the case of *Oxalis deppei*) the conclusion of Bassalik (1916) that oxalic acid originated in photosynthetic reduction.

Shibata (47), whose work was available to the reviewer only in the form of an abstract, also investigated the oxalic-acid content of various parts of *Begonia evansiana.* His method of micro-sublimation cannot be considered even as a reliable qualitative test and still less as leading to reliable conclusions concerning quantitative changes in plant organs.

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THE CHEMISTRY OF BACTERIA*

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Respiration

Warburg's yellow oxidation enzyme originally isolated from bottom yeast[1] has now been shown to be present and functional in the lactic-acid organism *B. delbrücki* (Warburg & Christian). This case is of special interest as the organism is an anaërobe and lacks catalase, but can oxidise certain sugars with production of hydrogen peroxide. This oxidation is independent of carbon monoxide and cyanide, and the peroxide formed has been shown to correspond to 70 per cent of the oxygen uptake. These facts have previously been used by Bertho & Glück[2] as a case of a living cell exemplifying the Wieland theory of oxidation, i.e., direct transport of hydrogen to oxygen with formation of hydrogen peroxide. It is now shown that the organism in question, though lacking cytochrome, possesses the yellow oxidation enzyme in unusually large amounts (77 mg. per kilogram of dry weight); activated hexose monophosphate is shown both *in vivo* (in presence of suspensions of *B. delbrücki*) and *in vitro* (in presence of the yellow oxidation enzyme) to reduce the pigment, which in its turn reduces oxygen and forms hydrogen peroxide; oxygen can be replaced by methylene blue, which the yellow oxidation enzyme alone reduces extremely slowly.

The nature of the green coloration produced by certain *streptococci* and the *pneumococci* on blood agar in which catalase has been destroyed by heat has now been reopened (Hart & Anderson). Up to the present the view of McLeod & Gordon that the green coloration is due to the action of hydrogen peroxide on haemoglobin has been widely accepted. The evidence used to support this view was that the organisms in question were known to produce hydrogen peroxide aërobically, but not in the presence of catalase, and a similar bleaching effect is produced by hydrogen peroxide on heated blood agar. The present authors distinguish between the bleaching effect due to peroxide and the formation of a green pigment. They have

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[1] Cf. *Ann. Rev. Biochem.*, **2**, 27 (1933).

[2] Cf. *Ann. Rev. Biochem.*, **2**, 499 (1933).

shown that the latter is produced anaërobically from laked blood, oxyhaemoglobin, methaemoglobin, and carboxy-haemoglobin by washed organisms in the presence of broth buffered at pH 7.0–9.0, with an optimum about 8.0. Autolysed cells can replace intact cells, but both require broth or meat extract. The cell factor is thermolabile, and is destroyed by oxygen and hydrogen peroxide; it is thus differentiated from the bleaching caused by the latter substance. The pigment was prepared as a dark green powder, insoluble in water, dilute acids, ethyl and amyl alcohol, ether and pyridine, but solube in 0.1 N NaOH, giving an olive green solution which is decolorised rapidly by hydrogen peroxide but not by reducing agents. The absorption spectrum has been examined, but the pigment awaits identification.

A respiratory study of the human tubercle bacillus (Loebel, Shorr & Richardson) aims at elucidating from studies *in vitro* factors which contribute to the maintenance of the organism in tuberculous lesions. The cultures were grown in Long's medium and then subjected to a period of starvation in saline and buffer in order to reduce the endogenous respiration to a minimum, after which they were removed to a respiration chamber, in which the oxygen uptake in various foodstuffs was measured and recorded in cubic millimeters per hour per milligram (wet weight) of bacteria. Of the constituents of the medium, glycerol was found responsible for ⅘ and the nitrogenous constituents for ⅕ of the total respiration. Glycerol is rapidly oxidised at about the same rate as lactate, oleate, palmitate, stearate, and lecithin, whilst serum and serum fat are oxidised at about twice this rate. Most of the substances tested produced a measurable respiration in low concentrations of the order in which they are found in blood. It is interesting that lactate is as readily oxidised as glycerol but fails to replace it in supporting growth.

Respiration in Long's medium was measured after various periods of starvation; after 15 days it was practically unimpaired, after which period it suffered a gradual decline, though it was still measurable even after 55 days' starvation.

The respiratory response to changes in hydrogen-ion concentration is remarkably small, no changes in respiratory rate being detected between pH 1.5 and 12.0 provided that the concentration of the acid was low (M/150). At higher concentrations of acid, respiratory inhibition was marked, M/50 phosphoric acid causing a 43 per cent inhibition, whilst M/15 lactic acid caused complete respiratory cessation.

Sensitivity to lack of oxygen is in striking contrast to tolerance in other respects. Respiration continues unimpaired after one day's exposure to anaërobic conditions, but after two days it is reduced to one-third and after three to four days it totally disappears, whilst the organism, when transferred to Long's medium and aërobic conditions, fails to grow. This is quite unlike the related organism, *B. phlei* (the timothy grass bacillus), which survives anaërobic conditions for 25 days with unimpaired respiration and maintains its viability for 30 days. Both organisms appear to lack an anaërobic glycolytic mechanism as measured by lactic-acid production from glucose, though a fermentative mechanism where acid is not the chief product is not excluded.

A series of papers on the kinetics of glucose oxidation by *Sarcina lutea* suffers from the defect that the rate of oxygen consumption is taken as a measurement of glucose oxidation *per se*, regardless of the fact that both aërobically and anaërobically the organism in contact with glucose constantly produces fresh oxidisable substances whose oxygen uptake complicates the issue. However, various important points are shown in the course of the work. Glucose is oxidised quantitatively to acetic acid and carbon dioxide; lactate, pyruvate, and glycerol are also oxidised; moreover, and very significantly, a sterile cell-free filtrate can be obtained which, though "manyfold" less active than the cells themselves, actually oxidises the same substrates (glucose, fructose, lactic acid, pyruvic acid, formic acid, and methyl alcohol) by molecular oxygen. Presumably such an extract contains several dehydrogenases, and at least one system activating molecular oxygen [Rubenstein (1932)].

Growth studies on the same organism [Rubenstein (1933)] show that a number of substances oxidised by the cell serve also as sources of carbon and energy, whilst others (e.g., acetate, formate, and methyl alcohol), though oxidisable, do not serve as the sole sources of carbon.

Acetate, though not alone supporting growth, adds to the amount of organism obtainable on glucose. Studies on salt requirements show the usual antagonism between Na, K, and Ca, the optimum ratio for a 3-chloride mixture being 8 Na : 1 Ca : 2 K. Phosphate and bicarbonate increase growth.

Azotobacter vinelandii has been shown to have an unusually high Q_{O_2}—up to 4,000—and to oxidise the following substances to completion (glucose, lactate, pyruvate, succinate, acetate, malonate, mal-

ate, tartrate, and fumarate) (Lineweaver). Respiration in this group is found to be a reversible function of pH with an optimum value at 7.2 and limits at 5 and 9; irreversible destruction occurs on 30 minutes exposure at pH 4.6 at 28°. Respiration is optimal at 34° with limits at 10° and 50°. The cessation of growth which has been often shown to occur with this organism at pH 5.8 is now seen to be due to inhibition of respiration partly reversible and partly irreversible. The catalase activity of the organism is high and optimal at neutrality, but still observable at pH 3.0, whilst at pH 4.0 the inhibition is still reversible and withstands a temperature of 92° for some hours. Exposure to hydrogen peroxide destroys it irreversibly. It is noteworthy that neither lack of oxygen nor of oxidisable material produces permanent injury, but merely a reversible inhibition of respiration (Burk, Horner & Lineweaver).

Further studies on the *gonococcus* (Barron & Hastings) have enabled the authors to study α-hydroxy-oxidase apart from the other oxidising systems of the cell. This mechanism has been separated, by heating to 55° for 2 hours, into two constituents termed by the authors "the activating co-enzyme," corresponding to lactic- or α-hydroxy-dehydrogenase, already known, and an oxidising enzyme equivalent to the *Atmungsferment*. A study of the kinetics of the system follows.

Experiments determining the concentration of cyanide necessary to inhibit growth in deep agar cultures of a number of aërobes and facultative anaërobes show wide variations with different organisms and different media. The results indicate great complication of factors, but no very definite conclusions are forthcoming (Braun & Guggenheim).

A study of the respiration of spores of certain aërobic bacilli has given some unexpected results [Tarr (1)]. It appears that spores of *B. subtilis, megatherium,* and *mesentericus* in the presence of glucose show an oxygen uptake of 20,000–43,000 cubic millimeters per gram of dry weight per hour. Where the respiration of spores and that of vegetative cells are compared the former is about 34 per cent of the latter. Heating spores to 80° for 30 minutes or incubating them aërobically increases their respiratory activity mainly by eliminating or shortening the latent period which otherwise occurs before maximum velocity is reached. Respiratory inhibitors show some unusual actions on spore respiration; cyanide, for example, has less effect on the respiration of spores than on that of vegetative cells—

M/500 KCN inhibiting the latter completely, and the former only to 70 per cent. Hydrogen sulphide (0.002 M) also shows anomalies, giving a 70 per cent inhibition with one strain of *B. subtilis* in the vegetative form and a 27 per cent acceleration of the spores of the same strain, whilst on spores of another strain of *B. subtilis* and of *mesentericus* and *megatherium* it has no action.

It is noteworthy that heating spores to 80° accelerates their subsequent respiration, the respiration itself exhibiting the same thermolability as the vegetative forms, though, in the case of spores, the heat-inhibition is reversible.

The study of the activation by *B. proteus* and *Staph. pyogenes aureus* of a number of compounds used as hydrogen donators and acceptors affords useful data in this field (Braun & Vásárhelyi). Data on the effect of agglutinins and amboceptors on dehydrogenating enzymes are included in this paper.

Acetic Bacteria

A study of the action of acetic bacteria (seven species) on lactic and pyruvic acids shows that the products from both acids are qualitatively the same, viz., acetylmethylcarbinol, acetic acid, and carbon dioxide. The authors (Hermann & Neuschul) suggest that the course taken is as follows: $CH_3 \cdot CHOH \cdot COOH \rightarrow CH_3 \cdot CO \cdot COOH \rightarrow CO_2 + CH_3 \cdot CHO \rightarrow CH_3 \cdot CO \cdot CHOH \cdot CH_3 \rightarrow CH_3 \cdot COOH$ No direct evidence for the production of acetic acid from acetylmethylcarbinol is adduced, but at the end of long experiments the latter substance tended to disappear and the former to increase. No experiments were done on the direct action of the organisms on acetylmethylcarbinol. *Bact. gluconicum* differs from the other species in forming no acetylmethylcarbinol.

Certain Japanese species of acetic bacteria produce kojic acid from fructose and mannitol (0.105–0.755 gm. of kojic acid from 10 gm. of fructose in 21–60 days) (Takahashi & Asai). Unlike the *Aspergillaceae*, these bacteria do not produce kojic acid from 3- or 5-carbon compounds. Of the five common vinegar species tested, one only (*Bact. xylinoides*) produces kojic acid, and this in very low yield.

Studies on oxidations by living suspensions of *Bact. pasteurianum* show that methyl alcohol is oxidised with an R.Q. of 0.57 (complete oxidation requires an R.Q. of 0.66); formaldehyde with an R.Q. of 0.78 (complete oxidation requires an R.Q. of 1.0);

and formic acid with an R.Q. of 0.67 (complete oxidation requires an R.Q. of 2.0). Acetone-treated organisms oxidise methyl alcohol, giving some formaldehyde but no formic acid (D. Muller).

Respiration can be shown to occur apart from growth by the use of bromoacetic acid at pH 6.0, which inhibits the latter at a concentration of 2.5×10^{-4}, whilst oxidation of glycerol continues unaffected till the concentration of bromoacetic acid reaches N/100 [Cozic (1)].

Growth can occur on ethyl alcohol and on acetate as sources of carbon, but not on methyl and isopropyl alcohols (Mosel).

It has been shown that the cellulose of *Acetobacter xylinum* can be synthesised from mannitol with extreme readiness, the cellulose formed amounting to 19.11 per cent of the mannitol used, whilst from sorbitol only 1.66 per cent was formed (Khouvine).

Bact. xylinum (Bertrand's sorbose bacillus) has been studied in great detail [Cozic (2)]. Findings of previous workers, notably Bertrand and Hermann & Neuschul, have been confirmed and extended, using modern methods. Two unexpected facts have come to light. Firstly, the organism, hitherto regarded as strictly aërobic, can develop anaërobically if oxygen is replaced by reducible dyestuffs such that their oxidised and reduced forms are in equilibrium at a suitable potential. Secondly, the respiration (aërobic) is strongly stimulated by cyanide (N/10–N/100), 35–40 mols. of cyanide disappearing during the uptake of 4–5 mols. of oxygen. The interpretation of this phenomenon is at present obscure. It is dependent on the presence of the enzyme catalysts (i.e., it does not occur with boiled organism), and it is more strongly marked in the presence of keto-bodies, e.g., dihydroxyacetone, but is still observable in their absence.

Fermentations

The biochemical properties of the group of fermenting *sarcinae* has been studied by Smit. This group includes *Zymosarcina ventriculi, Z. maxima,* and *Z. methanica.* All these are strictly anaërobic; the first was originally discovered in the human stomach in cases of ulceration and is, in addition, widely distributed in the soil. The resistance to mineral acids is remarkable, the organism withstanding nitric acid at pH 1.5, hydrochloric acid at pH 0.8, and sulphuric acid at pH 1.1.

The principal products of the fermentation of glucose (in per-

centage of sugar fermented) are: carbon dioxide, 41.7; ethyl alcohol, 40.3; acetic acid, 9.0; lactic acid, 3.0, and traces of formic acid and hydrogen. In the case of *Z. maxima,* butyric acid replaces ethyl alcohol, and succinic acid (3.4 per cent) appears.

Cl. butylicum decomposes hexoses according to the equation $C_6H_{12}O_6 = C_4H_8O_2 + 2H_2 + 2CO_2$. This reaction is much more sensitive to cyanide than lactic or alcoholic fermentations, being inhibited to the extent of 23, 60, and 100 per cent by concentrations of M/10,000, M/1,000, and M/100 respectively, a sensitivity of the same order as that of cell respiration. Unlike the latter, however, the inhibition of the butyric fermentation is not reversible by light; the author suggests that a metal catalyst other than iron may be at work (Kempner).

Data relating stereo-isomerism to fermentability (Koser & Saunders) show that bacteria which ferment the common (*l*-) form of arabinose ferment the *d*-form only after a delay (2–14 days). On the other hand, 3 strains of *proteus,* unable to ferment the *l*-form, ferment the *d*-form promptly. Also *Salmonella cholerae-suis* fails to ferment the *l*-form but ferments the *d*-form [Koser & Saunders (1)].

The introduction of the CH_3 group into the OH of the first carbon atom of various sugars stabilises the compound toward bacterial attack. Thus, whilst many bacteria ferment glucose, few only (including *proteus, Friedländer, aërogenes, pneumococcus,* some haemolytic *streptococci* and one yeast) ferment α-methyl glucoside, and α-methyl mannoside is not fermented by any organism known to ferment mannose. Other examples of this rule are given [Koser & Saunders (2)].

Data on the inhibition by the halogen derivatives of the fatty acids of lactic (Genevois & Nicolaieff) and acetic (Queré) fermentations are now available.

A study of the respiration and fermentation of *Lactobacillus pentoaceticus* shows that this organism differs from other lactic-acid bacteria in utilising oxygen more readily and in producing a higher proportion of acetic acid as compared with lactic acid. The oxidation is accelerated by oxygen carriers (methylene blue and pyocyanin) and is unaffected by cyanide. Sodium fluoride (0.01 N) inhibits strongly as does also monoiodoacetic acid (0.001 N) (Hunt).

Enzymes and Their Formation

Bacterial phosphatases have been demonstrated with growing and also with dried preparations of *Cl. acetobutylicum* and propionibacterium *jensenii* (Heard & Wynne and Pett & Wynne). A detailed study of hexosediphosphatase, α- and β-glycerophosphatase, and pyrophosphatase has been worked out with preparations of both organisms. In accordance with findings on mammalian phosphatase, Mg ions were found to have a very high accelerating effect on all three enzymes, whilst zinc activates pyrophosphatase only, and to a smaller extent. There is at present no evidence that the fermentation of hexose is associated with the formation of a hexose phosphate; attempts to demonstrate the synthesis of a hexose ester have so far failed.

The conditions governing the formation of the hydrogenlyases have been further examined (Yudkin). It will be recalled that formic hydrogenlyase is found in washed suspensions of cells when these are grown with broth and formate, but little or none when grown on broth alone. It is now shown that highly aërobic conditions operating during growth prevent the formation of lyases in all circumstances, though aërating a washed suspension of cells does not destroy the enzyme once formed. Working with a number of different bacterial species, it has now been found that, with few exceptions, when grown on simple inorganic media, with, e.g., glucose as source of carbon, no hydrogenlyases are formed. Some constituent of the broth, as well as the appropriate substrate for the hydrogenlyase, seems concerned in the formation of the enzymes.

Special experiments were conducted to prove whether the hydrogenlyases are produced during the 20-hour period of a culture as a result of natural selection [Stephenson & Stickland (2)]. It was shown that when formate is introduced into a growing culture of *Bact. coli* the enzyme appears in full strength before the lapse of a single generation, i.e., before any demonstrable increase in the total or viable count appears. Natural selection therefore cannot be operative as a cause of the enzyme formation, which seems rather to occur as a result of some interaction between the cells and the constituents of the medium.

Haines has extended his observations on the occurrence of gelatinase production on a synthetic medium. His findings, that Mg is essential to the enzyme formation, although negative or slightly antagonistic to growth, whilst Ca stimulates growth but is negative

with respect to gelatinase formation, have been extended to five organisms, and the phenomenon seems likely to be of general validity amongst bacteria.

Medium and Growth

Recent work has added several important items to our knowledge of the requirements of organisms which cannot support growth on inorganic forms of nitrogen and simple carbon compounds. It was found in the case of *B. diphtheriae* (Mueller, Porter, Klise & Graybiel) that the meat broth and peptone could be replaced by a 1 per cent acid hydrolysate of casein or edestin + 0.5 per cent Liebig's meat extract + 0.01 per cent of tryptophane, the latter therefore being an indispensable amino acid. The essential parts of the protein hydrolysate are:

1. Cystine (1 mg. per 10 cc.).

2. The proline fraction of the part soluble in butyl alcohol; this, however, is not replaceable by pure proline. The essential constituents of the Liebig's meat extract were highly concentrated but not identified. From 500 grams less than 1 gram of active material was obtained, of which less than 1 milligram per 10 cubic centimeters was necessary.

Information is at last coming in relative to the growth requirements of the *Clostridia* (Burrows). *Cl. botulinum* has been grown on an artificial mixture of amino acids of the following percentage composition: proline, 0.1273; glycine, 0.0075; leucine, 0.1617; cystine, 0.0083; lysine, 0.1303; and alanine, 0.5647; oxyproline and isoleucine can be substituted for proline and leucine respectively. Subcultivation was continued through three generations, but then failed, and growth generally was liable to cease for unexplained reasons. It is important to note that other members of the group, viz., *Cl. welchii, tetani, chauvei, hystolyticum,* and *sporogenes,* fail to grow on this medium.

An important factor in the cultivation of *Cl. sporogenes* has been brought to light by Knight & Fildes. These workers have separated from yeast an active substance, of which 2×10^{-7} grams support growth when added to 10 cc. of a basal medium consisting of inorganic salts, citrate, acid hydrolysate of casein, and thioglycollic acid. The properties and distribution of the "sporogenes vitamin" suggest a relationship to auxin. It was further shown that *B. aertrycke, B. tuberculosis,* and *A. versicolor,* which are themselves able to grow on

media lacking the vitamin, synthesise it during the process of growth, since alkaline hydrolysates of these organisms, grown in the absence of the vitamin, serve as a vitamin source for *Cl. sporogenes.*

Experiments on "training" an organism to dispense with constituents of its medium have been made on *Bact. typhosum* (Fildes, Gladstone & Knight). Four strains, which at the outset, in addition to a basal medium containing ammonia, citrate, and glucose, required leucine, lysine, and tryptophane, were found first to give delayed growth in the absence of leucine and lysine, and finally to dispense with them. These organisms were then "trained" to dispense with tryptophane by repeated subcultivation into media containing diminishing amounts of that amino acid. Two strains grown without tryptophane were tested with the glyoxylic reagent with positive results. Thus bacteria may be divided into three groups (Fildes & Knight):

1. Those requiring tryptophane in the medium, e.g., *Cl. sporogenes* and *Cl. botulinum.*

2. Those not requiring tryptophane in the medium, all autotrophic organisms and those able to grow on simple synthetic media.

3. Those ordinarily requiring tryptophane, but which can be "trained" to dispense with it, e.g., *B. diphtheriae, S. aureus, Bact. typhosum.* All organisms of whichever class, so far tested, give the glyoxylic test for tryptophane in the cell substance.

From meat extract a basic substance, active in a concentration of 1 milligram in 50 litres, has been obtained enabling *Staphylococci* to grow on a simple synthetic medium (Uschinsky's). A substance having similar properties was obtained from an alcoholic extract of *Bact. coli* (Hughes).

A study of the requirements of three organisms (colitis bacillus, Shiga-Kruse and Kruse-Sonne) shows that all do well on a synthetic medium with ammonia as the source of nitrogen. Various compounds as sources of carbon were tried, and various amino acids as sources of nitrogen and as sources of combined nitrogen and carbon. For the data obtained the original paper must be consulted. The inorganic requirements of the organisms were interesting; all three dispensed with Na, S, and Mg; two dispensed with K; all three required P (Bock).

The course of acetone fermentation by *Cl. acetoethylicum* has been shown to depend in a curious manner on the nutritional constituents of the medium. On the usual medium consisting mainly of

corn (maize) mash the carbohydrate is fermented giving both acetone and butyl alcohol. When grown on carbohydrate with peptone and the usual salts, butyl alcohol disappears from the fermentation products, but reappears if zein (present in corn mash) is added, though zein, in the absence of carbohydrate, gives rise to no butyl alcohol. Other proteins—corn globulin, egg albumin, glutin and casein—were ineffective in replacing zein (Weinstein & Rettger).

The Coloured Sulphur Bacteria

A contribution of prime importance (F. M. Muller) follows the work of van Niel reported last year.[3] It is now shown that this group of organisms can develop *anaërobically in light* on an inorganic medium with a number of simple substances as sources of carbon; such for example are lactate, pyruvate, acetate, succinate, malate, and butyrate, substances whose anaërobic decomposition is not accompanied by output of heat. What further distinguishes this remarkable mode of life is that growth occurs and the organic substrate is broken down with relatively very little evolution of carbon dioxide; that is to say, the substrate appears to pass into the cell material almost quantitatively. This is shown clearly from the following carbon balance sheet.

Medium	Substrate-Carbon Used	CO_2-Carbon Formed	Carbon in Bacteria	Total Carbon Recovered *per cent*
Lactate	3.29	0.41	2.95	102
	1.04	0.12	0.82	90
	3.70	0.25	3.15	92
Malate	4.48	1.25	2.72	89
	5.41	1.77	2.94	87
	2.92	0.86	1.93	96

With acetate, succinate, and butyrate the recovery was lower (63–92 per cent), but this was due to the difficulty of collecting the bacteria quantitatively. The striking point about these figures is the low carbon-dioxide loss. If the substrates are arranged in order of "oxidation values" (i.e. reducibility) butyric < lactic and acetic < succinic < malic. The carbon dioxide produced per millimol of substrate is in the reverse order: malate = 1.22; succinic = 0.7; acetic = 0.17; lactic = 0.29; butyric = −0.74. The last figure shows that butyric acid on passing into bacterial substance actually

[3] *Ann. Rev. Biochem.*, 2, 488 (1933).

takes up carbon dioxide; indeed, anaërobic growth of the bacteria on butyrate as sole source of carbon does not occur in absence of carbon dioxide. These findings accord with the view that in these anaërobic dehydrogenations carbon dioxide, as in the green plant, acts as the hydrogen acceptor, being provided partly by the bicarbonate of the medium and partly by the preliminary breakdown of the carbon compound. Possibly, since anaërobic development does not occur in the dark, the activation of the carbon dioxide is photo-catalysed. It is now even clearer than before that the coloured sulphur bacteria form a link with the plant world, the equation for photosynthesis being

$$CO_2 + H_2A \rightarrow H{\cdot}CHO + 2A + H_2O$$

H_2A being H_2O in the green plant, H_2S for the coloured sulphur bacteria in light when H_2S is present, and simple organic hydrogen donators for the same bacteria in light in the absence of H_2S.

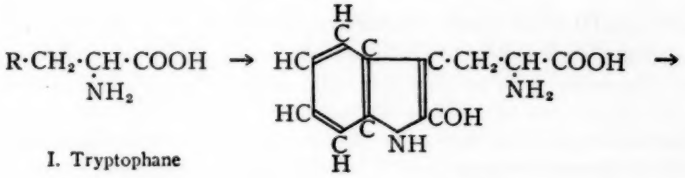
Amino-Acid Breakdown

Arginine has been found to be hydrolysed to citrulline by a mixed culture growing in the presence of peptone and glucose (yield about 8 per cent) (Ackermann). *B. pyocyaneus* in similar conditions—growth in the presence of lactate and ammonium phosphate—has now been found to bring about the same reaction (yield 4.9 per cent) (Horn).

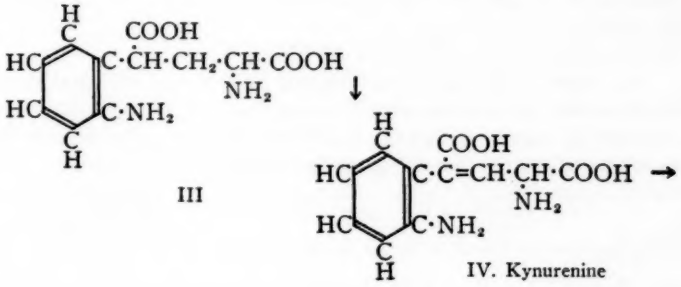
An important contribution to the breakdown of tryptophane by *Bac. subtilis* comes from Japan (Kotake & Otani). Heavy inoculations of the organism were made into a medium consisting of inorganic salts and 2.5 per cent of glycerol, and incubated for 40 days. In these conditions 2 grams of tryptophane gave 0.036 gram of anthranilic acid and 0.016 gram of kynurenic acid; 1.5 grams of kynurenine gave 0.27 gram of anthranilic acid and 0.021 gram of kynurenic acid. Estimations of kynurenine from tryptophane at varying intervals (10–30 days) showed that 0.3 gram of tryptophane gave the optimum yield (0.066 gm.) in 20 days. The suggested course of the reaction is as follows, the names of substances actually isolated being printed in italics.

It appears that indole lactic acid gives indole under the action of indole-producing strains of *Bact. coli* (Saito). Also that *d*-indole lactic acid, which probably corresponds to *l*-tryptophane, is more easily attacked than *l*-indole lactic acid.

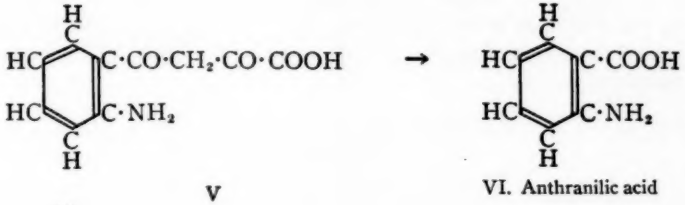
Using washed suspensions of *Proteus vulgaris* on a solution of



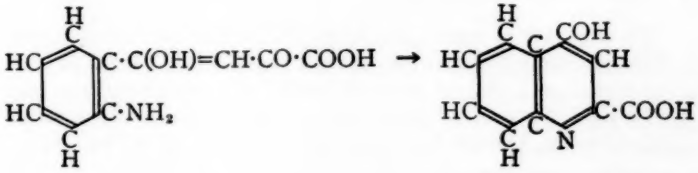
I. Tryptophane II



III IV. Kynurenine



V VI. Anthranilic acid



VII VIII. Kynurenic acid

l-cystine in buffer (500 mg. in 500 cc.) with full sterile precautions, Tarr (2) has shown that the decomposition occurs according to the equation:

$$\begin{aligned}\mathrm{COOH{\cdot}CH(NH_2){\cdot}CH_2S{-}SCH_2{\cdot}CH(NH_2){\cdot}COOH} &+ \mathrm{H_2} + 4\mathrm{H_2O} \\ = 2\,\mathrm{H_2S} + 2\,\mathrm{NH_3} + 2\,\mathrm{CH_3{\cdot}COOH} &+ 2\,\mathrm{H{\cdot}COOH} \\ &\downarrow \\ &2\,\mathrm{H_2} + 2\,\mathrm{CO_2}.\end{aligned}$$

The feature of this experiment is the use of thick washed suspensions and the consequent rapidity of the reaction uncomplicated by growth. The whole of the cystine was decomposed in 24 hours, and the yields of decomposition products lay between 90 and 100 per cent of theory.

Methane Formation

An organism has now been isolated by the single-cell technique which is able to live anaërobically on an inorganic medium with formate as sole source of carbon. It is shown that the reaction serving as source of energy for the growing culture is:

$$4\,\mathrm{H{\cdot}COOH} = \mathrm{CH_4} + 3\,\mathrm{CO_2} + 2\,\mathrm{H_2O} + 32\ \mathrm{Cals.}$$

This reaction is found to occur in two stages, viz:

$$(1)\ \mathrm{H{\cdot}COOH} = \mathrm{H_2} + \mathrm{CO_2} \qquad (2)\ 4\,\mathrm{H_2} + \mathrm{CO_2} = \mathrm{CH_4} + 2\,\mathrm{H_2O}$$

It is found that washed suspensions of the organism in addition to the reactions given above are able to bring about the strictly quantitative reduction of other 1-carbon compounds by molecular hydrogen.

$$\begin{aligned}\mathrm{H{\cdot}COOH} + 3\,\mathrm{H_2} &= \mathrm{CH_4} + 2\,\mathrm{H_2O} \\ \mathrm{CO} + 3\,\mathrm{H_2} &= \mathrm{CH_4} + \mathrm{H_2O} \\ \mathrm{H{\cdot}CHO}^{*} + 2\,\mathrm{H_2} &= \mathrm{CH_4} + \mathrm{H_2O} \\ \mathrm{CH_3OH} + \mathrm{H_2} &= \mathrm{CH_4} + \mathrm{H_2O}\end{aligned}$$

* Added as hexamethylene tetramine.

The organism is remarkable in its preference for 1-carbon compounds. Acetic, propionic, butyric, and caprylic acids, ethyl alcohol, acetaldehyde, and glucose were all tested in conditions where they formed the sole source of carbon for the organism, and also in broth cultures. In the former case no growth occurred; in the latter case, even where the organisms grew on the broth, they failed to produce methane from the added compound. In addition acetic acid, ethyl alcohol, and acetaldehyde were tested with washed suspensions in an atmosphere of hydrogen with negative results [Stephenson & Stickland (**2**)].

Inorganic Metabolism

Two remarkable organisms, both of which would seem to be of importance in soil economy, have been isolated from the neighbourhood of thermal springs at Aachen (Brussoff). In one case the slime in the neighbourhood of the spring was found to consist of masses of bacteria mixed with granules of silicic acid, which were also found within the cells. The organism, which was obtained in pure culture, closely resembles *Bac. mycoides*, and has been called *Bac. siliceus*. The organism appears to function as an agent for concentrating and depositing silicic acid, of which the spring water contains 0.0844 per cent, whilst the dried slime has 21.5 per cent.

In the same locality mud from a thermal spring was found to contain masses of calcium carbonate crystals; from this a bacterium was isolated, which, when grown on a medium containing 1 per cent of calcium chloride, deposited within the cell typical crystals of sphaerite.

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